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GONADOTROPHIC HORMONES AND OVARIAN HYPEREMIA IN THE RAT

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THE OVARIAN hyperemia reaction has been employed as a definitive endpoint in a rapid biological test for the early detection of pregnancy. This particular response of the ovary to urinary gonadotrophins was first noticed by Eberson and Silverberg in 1931 and is characterized by a congestion of the vessels of the ovarian tissue resulting in macroscopic reddening of the ovaries. The vasodilatation occurs soon after the administration of chorionic gonadotrophin and has been observed as early as one hour after intraperitoneal injection of urine of pregnancy (Kupperman, 1948). The hyperemic reaction while first described in the ovaries of immature albino rats has also been noted within six hours after the administration of pregnancy urine in the corpora lutea of ovaries of adult rats. A similar reaction was also observed in the ovaries of adult mice and immature hamsters (Kupperman et al., 1943; Kupperman and Greenblatt 1946-1948). The hyperemic effect noted in hamsters and adult mice was not consistently observed until 15 hours after subcutaneous or intraperitoneal administration of the urine. The reaction failed to develop or was not observed macroscopically in the ovaries of immature albino mice (Rockland strain) or immature or mature guinea pigs.

While the ovarian hyperemia response of the immature rat ovary to urinary gonadotrophins is generally accepted and has been employed with varying degrees of success as a test for the early diagnosis

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of pregnancy (Kelso, 1940; Frank and Berman, 1941; Salmon et al., 1942; Kupperman et al., 1943, 1948; Zondek, Sulman and Black, 1945; Riley et al., 1948) there is no agreement as to the factor or principle in the urinary gonadotrophins responsible for the production of ovarian hyperemia. In preliminary studies it was noted that this reaction occurred only after the administration of urine or extracts rich in luteinizing or luteotrophic hormones (Kupperman & Greenblatt, 1946). Extracts containing chiefly follicle stimulating hormone failed to induce ovarian hyperemia. These observations were substantiated by Zondek, Sulman and Black (1945) who believed that ovarian hyperemia is evoked mainly by the luteinizing hormone and that the follicle stimulating hormone has only an augmentative effect. On the other hand, Farris (1946) has reported ovarian hyperemia occurring after the administration of follicle stimulating hormone. In view of these dynamically opposite results, an extensive study was planned to note the role of various fractions of the gonadotrophic complex in the production of ovarian hyperemia. The following report is concerned with the effect of fractionated and unfractionated gonad stimulating extracts prepared from the pituitary gland, urine and serum on the induction of ovarian hyperemia in the immature female rat.

METHODS AND MATERIALS

Preparation of extracts: The follicle stimulating preparations used in this study were prepared by the tryptic-digestion method of McShan and Meyer (1940). The crude sheep pituitary gonadotrophic fractions (ISAP and Vetrophin-Abbott) and the cow pituitary fraction (IBAP140A) are the pH 5 soluble fractions that were recovered by precipitation with acetone and dried by washing with acetone or by lyophilizing according to the method reported by McShan and Meyer (1943). The luteinizing preparations (RLH and PLH) are the pH 5 and pH 4 insoluble fractions, respectively, and the purified gonadotrophic preparations (F) were obtained by methods reported by the same authors (McShan and Meyer, 1943). The lactogenic preparations (Lac and LacR) were made by the method of Bates and Riddle (1935). The purified horse gonadotrophin was obtained from E. R. Squibb & Sons, Incorporated, and the horse pituitary powder (AP17) was made by dehydrating the fresh glands with acetone. The rat pituitary gonadotrophin (RAP126) was obtained by aqueous extraction of air-dried (Kupperman, Elder and Meyer, 1941) rat pituitary glands. This extract was dried by lyophilizing. The purified pregnant mare serum gonadotrophin (PMS-E1P3) was prepared by the method of Cartland and Nelson (1937). The crude prolan preparations (ETOH and CPU105HM2) were obtained by alcohol precipitation of the chorionic gonadotrophin from human pregnancy urine. The thyrotrophic extract was prepared by the Armour Laboratories.[1]

Assay for ovarian hyperemia effect (six hours): The extracts to be tested were administered by the subcutaneous instead of the intraperitoneal route to remove the possibility that the solvents employed in the preparation of the extracts would cause irritative hyperemia by direct contact and thereby

[1] Supplied through the courtesy of Dr. F. F. Fenger.

produce a false positive reaction. While the extracts to be tested were administered in various doses, a constant amount of 2 cc. of aqueous diluent was always employed. The animals were sacrificed by ether asphyxiation six hours after administration of the hormone preparation. Twenty-five to 35 day-old female rats of a Wistar strain were employed. The viscera were exposed without trauma, and with no loss of blood. The ovaries were then examined for evidence of hyperemia and were classified as positive (those showing definite congestion), doubtful (ovaries exhibiting some slight vasodilation), and negative (no evidence of stimulation). The ovaries were permitted to remain exposed 2–3 minutes after autopsy to allow for a maximum expression of the hyperemic reaction.

Assay for gonad stimulating action (4.5 days): The extracts employed were assayed for their gonadotrophic effect by the administration of a given amount of the extract in 9 divided aliquots injected in 0.5 cc. portions once on the afternoon of the first day and twice daily for the next four days with autopsy performed on the morning of the 6th day. The extracts were administered in the above manner to 21 day-old intact female rats of the Sprague-Dawley strain. Qualitative and quantitative responses of the ovaries were noted.

While comparison between results obtained with the 4.5 day gonadotrophic assay and the six-hour hyperemic method could not be made on a quantitative basis, the comparative activity of the different preparations was established by each of the two assay methods. In addition the relative effectiveness of the various extracts in inducing ovarian hyperemia was compared on the basis of milligram equivalent of dried pituitary powder to the gonad stimulating effects of the same preparation.

RESULTS AND DISCUSSION

The data in Table 1 compare the gonad stimulating effect of different follicle stimulating hormone (FSH) preparations with the ovarian hyperemia inducing action of the same preparations. The extracts were all assayed in intact, non-hypophysectomized rats and proved to be principally follicle stimulating in action. Further assay of one preparation (FSH130) in 1 and 2.5 gram doses in immature male rats (Table 2) indicate that this extract exhibited no growth stimulating effect upon the prostate and seminal vesicles while increase in testicular weight was attained. These findings are in agreement with the prevalent concept of the dual action of FSH and luteinizing hormone (LH) on the seminiferous tubules and interstitial cells, respectively, and attest to the high degree of purification attained in the preparation of the FSH extract.

Analysis of the data presented in Table 1 indicate that at least 6-8 times the dose of FSH necessary to induce follicular stimulation associated with a 3-4 fold increase in ovarian weight is necessary before ovarian hyperemia may be produced. In addition findings are presented in Table 1 to show that FSH preparations less pure than others produce doubtful hyperemic responses in the ovaries in doses of 2 grams (FSH312 and FSH523). These findings imply that while ovarian hyperemia is not an expression of the FSH activity it may be

induced when sufficient LH is present in the administered preparation.

The effect of fractionated (other than FSH) and non-fractionated pituitary, urinary and serum extracts upon the induction of ovarian

TABLE 1. THE COMPARATIVE GONAD STIMULATING AND OVARIAN HYPEREMIA EFFECT OF FOLLICLE STIMULATING HORMONE (FSH) EXTRACTS

	Gonad Stimulating Action				Ovarian Hyperemia		
Preparation	Dose*	Number of Rats	Ovarian Weight	Ovarian Response	Dose*	Number of Rats	Reaction
FSH 130	0.5 gm. 1.0 gm.	3 3	43 89	All Follicles All Follicles	1 gm. 2 gm. 3 gm. 4 gm.	2 3 2 2	Negative Negative Doubtful Positive
FSH 129	0.5 gm. 1.0 gm.	9 3	53 85	All Follicles Few C. L.**	2 gm. 3 gm.	1 1	Negative Negative
FSH 312	0.5 gm.	3	94	Cloudy Follicles	1 gm. 2 gm. 3 gm.	2 3 1	Negative Doubtful-Negative Positive
FSH 313	0.5 gm.	6	61	All Follicles	2 gm. 4 gm.	1 1	Negative Positive
FSH 508	0.5 gm.	6	58	All Follicles	2 gm.	1	Negative
FSH 509	0.5 gm.	6	58	All Follicles	1 gm. 2 gm.	1 1	Negative Negative
FSH 522	0.5 gm.	7	42	All Follicles	2 gm. 4 gm.	1 1	Negative Negative
FSH 523	0.5 gm.	9	74	Few C. L.** in 3 rats All Follicles, otherwise	2 gm. 3 gm.	1 1	Doubtful Positive
FSH 5256	0.5 gm. .75 gm. 1.0 gm.	6 9 3	28 48 71	All Follicles All Follicles All Follicles	2 gm. 3 gm.	1 1	Negative Negative

* In grams equivalent of dried pituitary powder.
** A total 1–7 corpora lutea in both ovaries of each rat.

TABLE 2. THE EFFECT OF FSH 130 ON THE TESTES AND MALE ACCESSORIES OF THE IMMATURE RAT

Dose[1] (gram)	No. of Rats	Testes (mgm)	Seminal[2] Vesicles (mgm)	Prostate (mgm)
0	3	506	9.5	37.0
1.0	3	655	9.3	33.5
2.5	3	676	9.5	37.8

[1] Equivalent of dried pituitary powder.
[2] Less the coagulating gland.

hyperemia is presented in Table 3. These data indicate that in addition to the ovarian-hyperemic stimulating effect of unfractionated gonadotrophic extracts, highly purified LH and lactogenic (luteotrophic) extracts—in contrast to the FSH preparations—are also effective agents in producing ovarian hyperemia. The unfractionated aqueous extracts of both sheep and beef pituitary glands produce ovarian hyperemia in doses of 25 and 125 mg. equivalent, respectively. Quantitative assay of the beef pituitary extract over a period to 4.5

TABLE 3. GONAD STIMULATING AND OVARIAN HYPEREMIA EFFECT OF UNFRACTIONATED AND FRACTIONATED GONADOTROPHIC, LACTOGENIC AND THYROTROPHIC PREPARATIONS

Kind of gonadotrophic preparation	Gonad Stimulating Action				Ovarian Hyperemia		
	Total Dose*	Number of Rats	Ovarian Weight (mgm)	Ovarian Response**	Dose*	Number of Rats	Hyperemia
Crude unfractionated: Beef IBAP140A	500 mg. (+ 6 mg. Heme)	3	106	Several-Numerous Corpora Lutea	50 mg. 125 mg. 500 mg.	2 1 1	Negative Positive Positive
Sheep ISAP32A	100 mg.	9	52	Follicle-Numerous C. L.	10 mg. 25 mg. 50 mg.	3 2 1	Negative Positive Positive
ISAP24	100 mg.	6	94	Numerous C.L.	25 mg. 50 mg. 100 mg.	2 2 2	Positive Positive Positive
Vetrophin (Abbott)	100 mg.	6	93	Few C. L.	10 mg. 25 mg. 50 mg.	2 3 1	Negative Positive Positive
Horse AP17	15 mg.	3	97	Follicular Stimulation	5 mg. 10 mg.	3 2	Negative Negative
Rat RAP126	1 mg. 2 mg. 3 mg.	10 3 2	58 95 145	Several C. L. Several C. L. Several C. L.	0.5 mg. 1.0 mg. 2.0 mg. 3.0 mg. 4.0 mg.	2 3 2 2 2	Negative Negative Negative Negative Negative
Purified unfractionated: Sheep F115A	100 mg.	3	71	Few-Several C. L.	10 mg. 25 mg. 50 mg.	3 3 1	Negative Positive Positive
F116A	100 mg.	12	84	Follicles-Several C. L.	25 mg. 50 mg.	3 2	Negative Positive
Hog F119A	100 mg.	6	39	Follicular Stimulation	50 mg. 100 mg.	2 2	Negative Negative
Horse Gonadotrophin (Squibb)	2 units 5 units 10 units 15 units	3 3 3 3	21 84 261 337	Cloudy Follicles Numerous C. L. Many C. L. Many C. L.	1 unit 2 units 5 units 15 units	3 3 2 1	Negativ Positive Positive Positive
Pregnant Mare Serum PMSE1P3	0.25 mg.	3	85	Numerous C. L.	0.05 mg. 0.1 mg. 0.25 mg.	2 4 3	Negative Positive Positive
Chorionic PETOH	3 cc.	3	43	Few C. L.	3 cc.	2	Positive
CPU105	4 cc.	3	53	Few-Several C. L.	4 cc.	2	Positive
Fractionated sheep: Luteinizing hormone (LH) P118	500 mg.	3	16	Negative	50 mg. 100 mg. 250 mg.	2 3 1	Negative Positive Positive
R28A	500 mg.	1	15	Negative	100 mg. 250 mg.	2 2	Negative Positive
P116BC	500 mg.	2	13	Negative	25 mg. 50 mg. 100 mg. 250 mg.	3 2 2 1	Negative Positive Positive Positive
LH15	500 mg.	2	17	Negative	25 mg. 50 mg. 75 mg.	2 4 2	Negative Positive Positive
Lactogenic hormone LacR124	500 mg.	3	10	Negative	50 mg. 100 mg.	2 2	Negative Positive
LacR900	500 mg.	3	12	Negative	500 mg.	2	Positive
Lac52	500 mg.	2	10	Negative	500 mg. 1000 mg.	2 2	Positive Positive
Thyrotrophic hormone	5 units 10 units	3 3	13 14	Negative Negative	5 units 10 units	2 2	Negative Negative

* Mg. refers to mg. equivalent of dried pituitary powder.
** Few C. L. = A total of 1–7 corpora lutea in both ovaries
Several C. L. = A total of 8–14 corpora lutea in both ovaries
Numerous C. L. = Too many corpora lutea to count
Many C. L. = Ovary composed principally of corpora lutea

days according to the method described above in doses equivalent to 0.5 grams of dried powder failed to produce any detectable ovarian stimulation. However, as little as 125 mg. equivalent induced ovarian hyperemia. Incorporation of 6 mg. of heme with 0.5 gram equivalent of beef pituitary extract resulted in ovarian stimulation when the material was administered over a period of 4.5 days (McShan and Meyer, 1937). Luteinizing hormone extracts (P118, R28A, P116BC, LH15) caused ovarian hyperemia in doses as small as 100 mg. equivalent of dried pituitary glands. The LH preparations are 20-40 times as effective in the production of ovarian hyperemia as FSH when compared on the basis of equivalents of dried pituitary glands. These same luteinizing extracts were ineffective in exhibiting gonad stimulating action when administered over a 4.5 day period to the immature 21 day-old female rat. However, when the LH preparations were administered in conjunction with FSH extracts, corpora lutea were produced. Lactogenic preparations were effective in producing ovarian hyperemia in doses which failed to show evidence of gonad stimulating activity (Table 3). One preparation (LacR124) induced ovarian hyperemia in doses equivalent to 100 mgm. of dried pituitary powder and compares favorably with the similar activity of LH preparations.

Pregnant mares' serum, human pregnancy urine extracts and commercially available unfractionated sheep pituitary preparations all possessed LH activity and readily produced ovarian hyperemia. Administration of a thyrotrophic extract and suspension of dried horse pituitary failed to induce hyperemia of the ovarian tissue. The thyrotrophic preparation exhibited no gonad stimulating action in the immature rat and the crude horse pituitary extract showed only follicular stimulating activity in the doses employed. Hence it may be concluded that the absence of sufficient LH in these preparations prevented the induction of ovarian hyperemia.

Our results have consistently demonstrated that extracts possessing LH or luteotrophic activity are capable of producing ovarian hyperemia. In addition it may be noted that while highly purified FSH extracts are practically ineffective in producing ovarian hyperemia, FSH enhances the ovarian hyperemic action of LH. This is supported by data in Table 3 where it is shown that unfractionated gonadotrophic extracts (ISAP24) are as a rule more effective in producing ovarian hyperemia than LH preparations when a comparison between the minimal hyperemic stimulating dose (on basis of milligram equivalents of dried pituitary powder) is made. The augmentative effect of FSH in the presence of LH upon ovarian hyperemia was also demonstrated by Zondek et al. (1945). The ovarian hyperemic stimulating action of lactogenic hormone may be ascribed to its luteotrophic properties since it has been shown that lactogenic preparations will maintain the function of the corpus luteum (Astwood, 1941; Evans,

Simpson and Turpeinen, 1938). On the other hand, gonad stimulating extracts which are predominantly follicle stimulating in action fail to induce ovarian hyperemia except when excessive doses of these preparations are employed. There is, however, one exception that we have noticed to the above concept that LH preparations will produce ovarian hyperemia, namely it was observed that when a gonadotrophic extract prepared from rat pituitary glands was administered to immature female rats ovarian hyperemia failed to develop. Ovarian hyperemia was not observed despite the fact that doses far exceeding the minimal dose necessary to produce ovarian hypertrophy and corpora lutea formation (4.5 day assay) were employed. Although no explanation is at hand to account for the failure of rat pituitary extracts to induce ovarian hyperemia in the rat, it is possible that homologous pituitary preparations are not hyperemic in action notwithstanding their LH activity.

The hyperemic inducing effects of LH and luteotrophic extracts suggest the use of this end-point as a rapid assay method for establishing luteinizing and luteotrophic activity of unqualified gonadotrophic preparations. Such a procedure may serve as a means of rapidly screening a large number of compounds in testing for such activity. The ovarian hyperemia response may also be employed in quantitative assays for LH or luteotrophic hormone in the serum of normal and pregnant women and in those patients presenting suspected pathological findings such as chorionepithelioma and hydatidiform mole when greatly increased levels of urinary gonadotrophins are present. In addition it has recently been suggested (Zondek et al., 1948) that diagnosis of fetal death may be made by noting quantitatively the ovarian hyperemia stimulating action of various dilutions of human pregnancy urine. The greatest dilution at which ovarian hyperemia is attained may be used as the end-point for quantitative assays. Thus the hyperemia unit (OHU) may be defined as the least amount of material or greatest dilution of serum or urine which will produce ovarian hyperemia in all of the animals injected six hours after subcutaneous administration of the preparation to immature female rats, 25-30 days of age and 45-70 grams in weight.

While our earlier results (Kupperman and Greenblatt, 1946) and the present observations are in agreement with the findings of Zondek, Sulman and Black (1945), we are at a loss to explain the discrepancies between our findings and those of Farris (1946). This latter worker has ascribed the hyperemic response of the rat ovary to gonadotrophins as due to the FSH activity of the preparations. While we have employed the same end-point, different methods of asphyxiation of the test animal have been used. Whereas ether has been the lethal agent in our experiments, Farris has adopted the use of illuminating gas. Perhaps the use of such an agent could sufficiently alter the appearance of the ovaries to explain the difference.

SUMMARY

Ovarian hyperemia has been employed as a definitive end-point in the rapid (2-6 hours) pregnancy test utilizing the rat as the test animal. Studies were undertaken to ascertain the gonadotrophic factor responsible for evoking ovarian hyperemia. The following preparations from pituitary glands, serum and urine were tested: Unfractionated pituitary extracts and fractionated pituitary preparations i.e. follicle stimulating hormone, luteinizing hormone, lactogenic and thyrotrophic extracts; pregnant mare serum, and pregnancy urine extracts. Some pituitary preparations were administered as aqueous suspensions of dried pituitary powder. Only those preparations exhibiting luteinizing or luteotrophic activity were capable of inducing ovarian hyperemia when administered according to the described procedure. A comparison between the hyperemic action and the gonadotrophic potency of the various extracts was made. In view of the specificity of the luteinizing and the luteotrophic hormones in evoking ovarian hyperemia it has been suggested that this end-point be employed as a rapid procedure in screening gonadotrophic compounds for their luteinizing and luteotrophic effects. An ovarian hyperemic unit is described and its use in an assay method is discussed.

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THE CRITICAL REQUIREMENT FOR PANTOTHENIC ACID BY THE ADRENALECTOMIZED RAT[1]

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INTRODUCTION

IN EARLIER experiments it was observed that survival in adrenalectomized rats was significantly prolonged when the diet was supplemented with very large doses of pantothenic acid and the rats were allowed 1% NaCl as drinking water (Ralli, 1946). In view of this finding we were interested in determining the critical dose of pantothenic acid necessary for the prolonged survival of rats following adrenalectomy. This report is concerned with the effect of daily doses of pantothenic acid varying from 0.03 to 6 mg. on survival in adrenalectomized rats.

EXPERIMENTAL

Black rats of the Long-Evans strain, bred in the laboratory, were placed on a diet deficient in pantothenic acid (Ralli and Graef, 1943) when 30 days of age. This diet contained 22% vitamin-free casein, 64% sucrose, 9% Primex, and 5% of the modified salt mixture[2] containing no NaCl. 100 gm. of the basal diet was supplemented with 1.9 cc. cod liver oil, 0.3 mg. thiamine hydrochloride, 0.3 mg. pyridoxine hydrochloride, and 0.9 mg. riboflavin. The deficient diet was continued for 30 days, by which time the failure of the rats to gain weight, graying of the fur, and the small excretion of pantothenate in the urine[3] all indicated that depletion of pantothenic acid had occurred. The rats were then adrenalectomized. Following adrenalectomy the diet was supplemented daily with the dose of calcium pantothenate to be tested. These dosages will be presented as the amount of pantothenic acid equivalent to the dose of calcium pantothenate given. In order to avoid unnecessary manipulations of the animals, and also to distribute the dose of pantothenic acid throughout the day, 0.5 ml. of a solution of calcium pantothenate (made up to contain 2 daily doses

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[2] The composition of the modified salt mixture was 2 gm. ferric citrate, 38 gm. calcium diphosphate, 36 gm. potassium citrate, 10 gm. magnesium citrate, 0.04 gm. copper sulphate, and 0.005 gm. potassium iodide.

[3] We are indebted to Dr. Saul H. Rubin, Director of the Nutrition Laboratories, Hoffmann-LaRoche, Inc., Nutley, New Jersey, for the pantothenate assays of rat urine.

per ml.) was mixed with the amount of food each rat was expected to eat during the next 24 hours. Food consumption records were kept for all animals and the total quantity of food offered was adjusted to each rat's appetite. Throughout the survival experiments all rats received a 1% solution of NaCl as drinking water. The rats were weighed at frequent intervals.

Some rats in each group were deprived of salt at intervals throughout the experiment in order to test the completeness of adrenalectomy. Other animals which had survived adrenalectomy for long periods were subjected to stress in order to evaluate their condition. These rats were obliged to swim for 25 minutes in water at 25° C. Blood sugar was determined in tail blood before and after swimming by the method of Polis and Sortwell (1946).

RESULTS

Table 1 shows the effects on survival after adrenalectomy of varying doses of pantothenic acid daily. In all experiments in which the

TABLE 1. EFFECT OF PANTOTHENIC ACID ON SURVIVAL IN ADRENALECTOMIZED RATS

NaCl	Pan. acid daily mgm.	No. of rats	Per cent of total surving Days 10	25	50	75	100	150	Mean S.D.
1% in water	0	105	50	2	0	0	0	0	11.9 ±5.4
	0.03	19	74	0	0	0	0	0	14.2 ±4.3
	1.0	15	87	7	0	0	0	0	
	2.0	14	93	43	36	29	29*		
	3.0	31	74	16	16	16	16*		
	3.5	14	71	43	43*				
	4.0	22	96	77	73	50	41*		
	4–6	70	100	91	81	67	62	50	
None	0	26	7	0					6.2 ±2.5
	4–6	21	62	0					12.5 ±4.5

* Experiments discontinued . . . animals used for special studies.

rats received up to 4 mg. of pantothenic acid daily, the calcium pantothenate was administered to each rat in the amounts indicated in the table. The animals indicated as receiving 4 to 6 mg. of pantothenic acid daily were supplied *ad lib.* with a diet containing 4 mg. of calcium pantothenate in 10 gm. of diet. The amount of diet consumed daily was weighed during periods of the experiment and, although the daily intake of the diet varied, most rats ingested an average of about 5 mg. of pantothenic acid per day (Ralli, 1946). The control group consisted of adrenalectomized rats which were continued on the deficient diet after adrenalectomy.

The necessity for NaCl has previously been reported (Ralli, 1946). Without salt, the rats survived only a few days after adrenalectomy, whether or not they were receiving pantothenic acid. The survival for the rats without salt is also given in the table.

The results show that survival following adrenalectomy was sharply curtailed in animals continued on the deficient diet and 1% NaCl. When 0.03 mg. of pantothenic acid daily was fed, the mean survival was slightly longer, but survival was still strictly limited. This amount of pantothenic acid has been shown to be more than

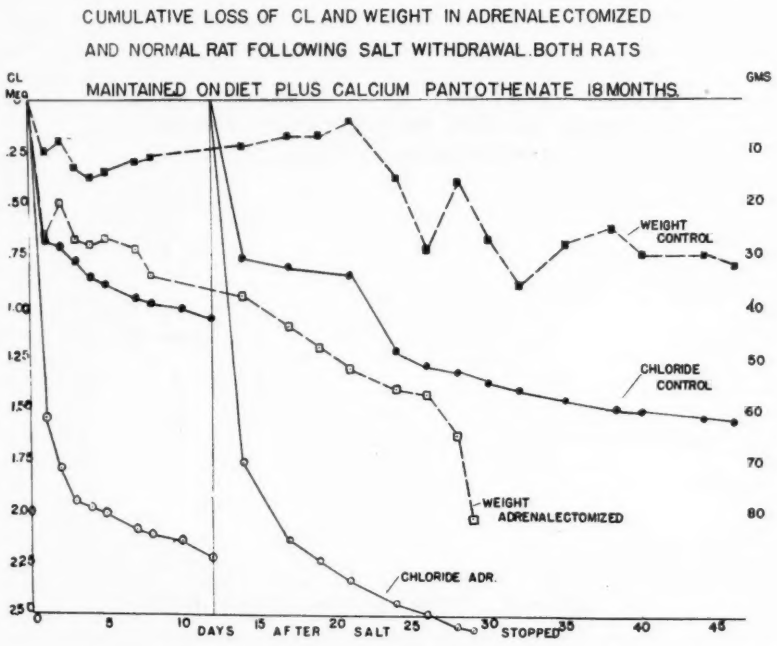

FIG. 1. Effect of salt withdrawal in an adrenalectomized and a normal rat. The 2 rats were placed on a diet deficient in pantothenic acid when 36 days of age. After 30 days on the diet one rat was adrenalectomized and the other rat was sham operated, and in both rats the diet was supplemented with calcium pantothenate (4 to 6 mg.) and a 1% NaCl solution. After 18 months NaCl was stopped. At this time the adrenalectomized rat weighed 272 gms. and the control animal weighed 207 gms. The adrenalectomized rat survived salt withdrawal for 28 days and during this time it lost 51 gms. in body weight. The control rat during the same period of time lost 22 gms. The control rat continued to survive and was finally sacrificed after 6 weeks. The line at 12 days indicates a break in the experiment when through an error NaCl was administered for a short period of time.

sufficient for optimal growth by intact rats 10 weeks of age (Unna and Richards, 1942). On 1 mg. of pantothenic acid daily a larger percentage of the animals survived for 10 days, and one animal (7%) survived for 25 days. The completeness of adrenalectomy in this animal was checked by withdrawing salt and the animal succumbed within 26 hours.

When the dose of pantothenic acid was increased to 2 mg. or more

daily, there was a definite increase in percentage survival, and in some of each group prolonged survival was obtained. On 2, 3, and 3.5 mg. daily less than half of the rats survived for 25 days or more. When the daily dose of pantothenic acid was increased to 4 mgm., 77% of the rats lived for 25 days or more. When the rats ingested the diet *ad lib.* and the daily intake of pantothenic acid varied from 4 to 6 mgm., 91% of the animals survived adrenalectomy for more than 25 days.

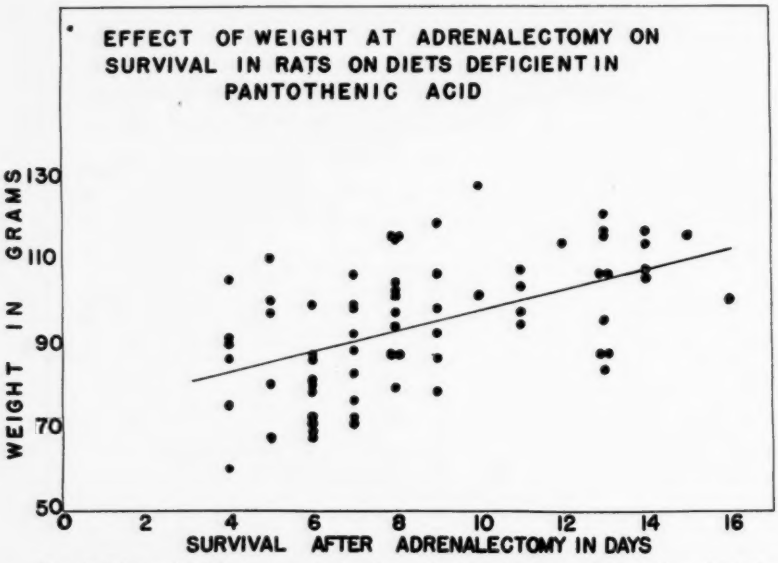

FIG. 2. The weight in grams at the time of adrenalectomy is plotted against the survival after adrenalectomy in days. All rats had been maintained on a diet deficient in pantothenic acid for 30 days before adrenalectomy and were continued on the same diet plus 1% NaCl after adrenalectomy.

The completeness of adrenalectomy was checked in many of the long-surviving animals by depriving them of NaCl. All of the rats tested succumbed to salt deprivation, usually within 2 to 4 days. An occasional rat which had been maintained on NaCl and pantothenic acid for a long time following adrenalectomy withstood NaCl deprivation for a much longer period. While the completeness of adrenalectomy might be questioned in these cases, both the weight and chloride losses were much greater in the adrenalectomized rats than in their intact controls on the same regime. Figure 1 shows the cumulative loss of weight in grams and of chloride in milliequivalents of such an adrenalectomized rat compared with its intact control after salt was withheld from both. The adrenalectomized rat had survived adrenalectomy for 18 months at the time salt was withdrawn. This animal survived adrenalectomy for an unusually long period and also sur-

vived salt withdrawal for a total of 28 days. The survival following salt withdrawal was influenced by a break in the experiment when through an error salt solution was given to both rats, and this, as indicated in the chart, occurred on the 12th day. Withdrawal of salt again was associated with a sharp drop in weight in the adrenalectomized rat and in a negative chloride balance. In the intact control there was a loss of weight, but this became stabilized after the animal had lost approximately a total of 30 gm. A similar loss of chloride and weight was reported (Ralli, 1946) in other animals surviving adrenalectomy for prolonged periods.

Analysis of the data on 105 control animals which were continued on the deficient diet following adrenalectomy showed that the weight

TABLE 2. THE SURVIVAL INDEX* OF ADRENALECTOMIZED RATS

Pantothenic acid mg./day	Number of rats	Mean weight at adr. gms.	Median survival after adr. days	Survival* index
0.0	10	95	8	1.1
0.0	15	102	8	0.9
0.0	11	80	6	0.9
0.03	19	120	12	1.0
1.0	15	83	13	1.9
2.0	14	80	20	2.9
3.0	31	85	15	2.1
3.5	14	98	18	2.2
4.0	22	100	76	9.5
4–6	70	103	150	16.7

* The survival index is the ratio of the median survival of rats on a given intake of pantothenic acid to the expected survival of rats of the same weight ($\pm$10 gms.) on a diet deficient in pantothenic acid.

of the rat at the time of adrenalectomy is one factor affecting its survival following the operation. This data is shown in Figure 2 in which the weight of the rat is plotted against its survival after adrenalectomy. The regression line was calculated by the method of least squares. This same tendency for the heavier animals to survive longer after adrenalectomy than the lighter ones could be demonstrated in all groups of rats except those receiving 4 to 6 mg. of pantothenic acid daily.

Since weight is one factor influencing survival after adrenalectomy, we wished to express the survival of rats receiving various doses of pantothenic acid in a way which allowed for this variable. We have, therefore, devised a survival index based on the mean weight of the animals in each group at adrenalectomy. The survival index is the ratio of the median survival of rats on a given intake of pantothenic acid to the expected median survival of rats of the same weight, $\pm$ 10 grams, on a diet deficient in pantothenic acid. Table 2 shows the survival index for each group of adrenalectomized rats with reference to the dose of pantothenic acid received. As a partial check on the

validity of this procedure, the survival index has been calculated separately for each of 3 small groups of control animals chosen at random. All give a result close to the theoretical value of 1.0. Rats receiving 0.03 mg. of pantothenic acid daily also showed a survival index of 1, indicating that the slight extension of survival on 0.03 mg. shown in Table 1 was probably due to the fact that this group of

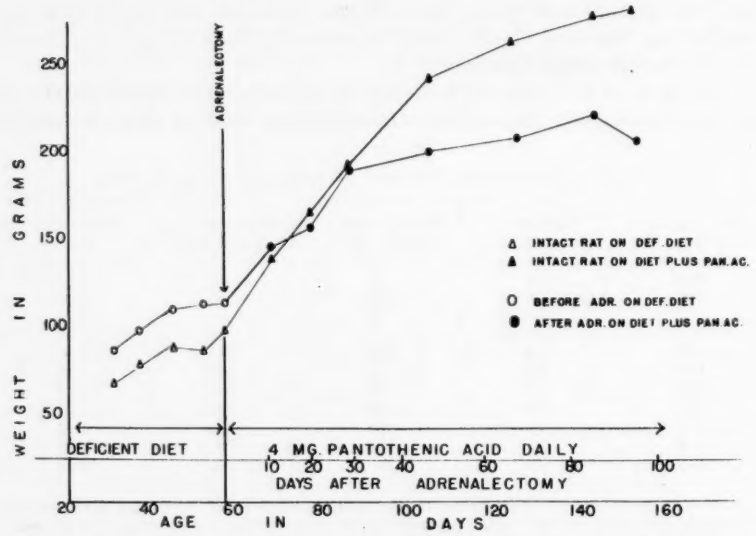

FIG. 3. Comparison of growth curves in adrenalectomized and intact rats on 4 mgm. of pantothenic acid daily. Average growth curves of 4 intact and 4 adrenalectomized male rats. All rats were placed on a pantothenic acid deficient diet when 32 days of age and were continued on the diet for 26 days. At this time 4 of the rats were adrenalectomized and 4 were continued as intact controls and each rat received 4 mgm. of pantothenic acid daily.

animals was unusually heavy. Intakes of 1, 2, 3 and 3.5 mg. of pantothenic acid daily approximately doubled the expected median survival, and therefore doubled the survival index, giving values ranging from 1.9 to 2.9. On 4 mg. of pantothenic acid daily the survival index increased strikingly to a value of 9.5 and on 4 to 6 mg. the survival index was 16.7. In other words, while a few individual rats may survive adrenalectomy for prolonged periods on diets providing up to 3.5 mg. of pantothenic acid daily, 4 mg. or more of pantothenic acid are required for any marked extension of survival in more than half the animals.

When adrenalectomized rats were fed large doses of calcium pantothenate they not only survived but appeared well and grew almost normally for a time. The maximum weight attained was, however, consistently less than that of intact rats on the same diet. In Figure 3

average growth curves are shown for 4 intact male rats and for 4 adrenalectomized male rats. All the animals were placed on the pantothenic-acid deficient diet when 32 days of age. Half the animals were adrenalectomized at 58 days of age and all received 4 mg. of pantothenic acid daily from 58 days until the conclusion of the experiment. Each point represents the mean weight of 4 rats at the indicated age.

For about a month after adrenalectomy the intact and adrenalectomized rats kept pace with each other fairly closely. The temporary interruption in the rate of growth which occurred in the adrenalectomized rats between 10 and 20 days after adrenalectomy is characteristic

TABLE 3. EFFECT OF SWIMMING ON BLOOD SUGAR

Condition of rat	No. of rats	Diet or supplement	Initial blood sugar mg. %	Final blood sugar mg. %	% Change in blood sugar
Intact	4	Nuchow	134 ± 7	112 ± 11	−16 ± 9
2 Days after Adrenalectomy	3	Nuchow	133 ± 10	93 ± 12	−31 ± 12
6 Days after Adrenalectomy	4	Nuchow	138 ± 11	95 ± 9	−32 ± 6
Intact Rat on Exp. Diet for 100 Days	3	4 mg. Pan. per day	125 ± 9	133 ± 20	+ 6 ± 13
Adr. Rats 100 Days after Adr. on Exp. Diet	3	4 mg. Pan. per day	136 ± 7	122 ± 5	−11 ± 2

and has occurred consistently. It seems to reflect a critical period in the adjustment of the adrenalectomized rat to the loss of its cortical hormones. Animals on an inadequate intake of pantothenic acid were likely to die during this period, and those which survived usually continued to survive for relatively long periods.

About 30 days after adrenalectomy the rate of gain of the adrenalectomized animals dropped below that of the intact animals. The maximum weight of the adrenalectomized rats was reached about 80 days after adrenalectomy, when the rats were 140 days of age. At this time the average weight of the adrenalectomized rats was 220 grams, while the intact rats weighed an average of 275 gm. and were still gaining.

Similar curves have been constructed using data from female rats. The relationship between the weights of adrenalectomized and intact rats was similar to that found in males, although the absolute weights were lower.

It should be emphasized that these adrenalectomized rats receiving adequate amounts of pantothenic acid and NaCl appeared well and, as will be shown below, were capable of muscular work.

Since the ability of an animal to withstand stress is considered a significant indication of adrenal cortical function, we have subjected adrenalectomized and intact rats to swimming as a form of stress. The rats were placed in large stone crocks containing water at 25° C and were obliged to swim for 25 minutes. Tail blood was taken for the determination of blood sugar before and immediately after swimming. The ability of a given animal to withstand stress was judged by the effect of the swim on the animal's blood sugar.

The data are summarized in Table 3, where the initial and final blood sugar values are shown for intact and adrenalectomized rats on Nuchow, and for intact and adrenalectomized rats on the experimental diet providing 4 mg. of pantothenic acid daily. The values shown are the means and standard deviations of the means.

The data show that the initial blood sugar values were essentially the same for all groups of animals tested, but that their ability to maintain this value after swimming varied widely. The intact animals receiving 4 mg. of pantothenic acid daily showed a slight, though probably insignificant, rise in blood sugar following swimming. The adrenalectomized rats on the same diet showed a moderate decrease of 11%, which was less than the 16% loss in the intact Nuchow rats and very much less than the 30% loss in adrenalectomized rats on Nuchow.

DISCUSSION

These observations point to the importance of pantothenic acid in the diet of adrenalectomized rats. The amount required is so large in comparison with the dietary needs of intact rats that it seems unlikely that its action is merely one of overcoming a deficiency in pantothenic acid.

The adrenalectomized rats on large doses of pantothenic acid were in good physical condition. They appeared well and could not be easily distinguished from the intact animals. The fur in both the intact and adrenalectomized rats was thick and well pigmented. The adrenalectomized animals were somewhat less active than the intact animals and their food intake was slightly less. For a time there was little difference in weight between the 2 groups but ultimately the intact rats outstripped the adrenalectomized rats. By the 100th postoperative day there was a considerable difference in the average weight of the 2 groups.

The stress experiments showed that these adrenalectomized animals were in unusually good condition for rats 100 days after adrenalectomy which had received no hormone therapy at any time. Pantothenic acid prevented the fall in blood sugar which would have been expected in adrenalectomized rats subjected to stress and which was observed in the adrenalectomized rats on Nuchow. One reason for the prolonged survival of these animals may be that large doses of pantothenic acid protect rats from the disturbance in carbohydrate

metabolism which ordinarily follows adrenalectomy. It has been postulated repeatedly (Williams, 1941; Wright, 1942; Berkman, Dorfman and Koser, 1942; Hills, 1943) that pantothenic acid is related in some way to the carbohydrate metabolism of normal animals, possibly through participation in a coenzyme system (Lipmann et al., 1947; Nachmansohn and John, 1945). The effectiveness of pantothenic acid in maintaining life in the adrenalectomized rat may be associated with its action on carbohydrate metabolism.

Another way in which pantothenic acid may be contributing to survival in the adrenalectomized animal is in maintaining water balance. Gaunt and his associates (1946) showed that pantothenic-acid deficient rats were very sensitive to large doses of water and that water intoxication was prevented by the administration of either pantothenic acid or adrenal cortical extract. The combined administration of NaCl and pantothenic acid to adrenalectomized rats may have compensated in some way for the loss of the adrenal hormones in respect to water and salt metabolism.

The physiological disorder caused by adrenalectomy is not confined to a single function, and pantothenic acid without NaCl is ineffective—but NaCl is equally ineffective in maintaining survival of adrenalectomized rats on diets deficient in pantothenic acid. Our experiments did not define the role of pantothenic acid in the adrenalectomized rat, but the amount of pantothenic acid necesary for survival is now established and its importance is again demonstrated.

SUMMARY

Black rats 30 days of age were placed on a diet deficient in pantothenic acid. After 30 days on the deficient diet the animals were adrenalectomized. The adrenalectomized rats received the same diet plus graded amounts of pantothenic acid ranging from 0.03 mg. to 6 mg. daily. Throughout the experiments, the rats were given 1% NaCl as drinking water.

Prolonged survival following adrenalectomy was observed in more than half the rats receiving 4 mg. or more of pantothenic acid daily. A few animals receiving from 1 to 3 mg. of pantothenic acid survived adrenalectomy for prolonged periods.

The weight of the rat at the time of adrenalectomy was shown to be one factor influencing the subsequent survival of animals receiving 3.5 mg. or less of pantothenic acid.

Adrenalectomized rats receiving 4 mg. or more of pantothenic acid daily grew normally for about 4 weeks postoperatively. Thereafter the intact rats outstripped the adrenalectomized animals.

Rats 100 days after adrenalectomy, maintained on 4 mg. of pantothenic acid daily, showed less decrease in blood sugar following swimming than either intact or 6-day adrenalectomized rats on Nuchow.

ACKNOWLEDGMENT

We wish to acknowledge the assistance of Mr. Paul Roth in the stress experiments.

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A SIMPLIFIED TECHNIQUE FOR HYPOPHYSECTOMY OF THE DOMESTIC FOWL

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THE SURVIVAL of adult chickens following hypophysectomy by the transbuccal method has been found, in my own experience, to be comparatively low, though not as low as has been reported by Hill, Corkill and Parkes (1934). The first week after hypophysectomy, in particular, is the period of most severe losses. Since the trauma associated with the operation seemed to be primarily responsible for these losses a less traumatic technique was looked for; this was found in the oral approach[2] to the pituitary, which White (1933) used successfully on rabbits. This approach makes for a faster, simpler, and cleaner operation than the one using the transbuccal approach. Early mortality (in the first to second postoperative weeks) was considerably less (Rothchild, 1948) than that following the transbuccal method, although total mortality was practically the same by both methods.

The details of the method follow:

Equipment. The operating board is shown in perspective view in figure 1, and with its legend, is self-explanatory. The instruments needed (in addition to a dental drill, #8 or #10 excavating burrs, suction cannulas, and a suction pump) are a pair each of fine straight, and fine curved forceps, a small probe, a bone scraper (such as a dental discoid excavator), a pair of spring steel spreaders (shown in use in figure 2), a dissecting probe with end hooked and sharpened, and a spatula about 1 mm. in width.

Anesthesia. Nembutal (6.0% in 10% alcohol) by the intravenous route is used as the sole anesthetic. The effective dose is about 0.35 cc. per kilogram of body weight. Slightly over half of the total amount required should be given in a single quick thrust of the plunger and the remainder in 0.05 cc. amounts, with the speed of administration roughly in proportion to body weight. The best criterion of adequate anesthesia is a slight head twitch on pinching the comb. The last 0.10–0.15 cc. should in all cases be given slowly and carefully, with comb pinches between each 0.05 cc.

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[2] The oral approach was evidently also used by Mitchell (1929), but he gives no details about it.

The operation. The bird is laid, back down, on the operating board, the comb fitted into the comb slot, the neck between the upright prongs of the head holder, and the upper half of the beak into the beak holder (see figures 1 and 2). The bird's head is adjusted to a horizontal position, the rubber band is slipped under the lower jaw, with the tongue elevator under the tongue, and then fixed to the ap-

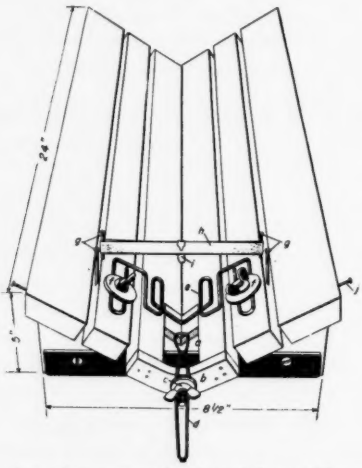

FIG. 1. Perspective view of the operating board for oral hypophysectomy: *a*—comb slot (3×¾ inches); *b*—V-shaped piece of wood closing forward end of comb slot and serving as support for *c*—wing nut, bolt and washer; *d*—beak holder; *e*—head holder (made of heavy copper wire—the height of the upright prongs should be about 1¼ inches); *f*—wing nut, washer and bolt for clamping lateral arms of the head holder; *g*—pegs or nails for supporting *h*—rubber band for retraction of the lower jaw; *i*—wing or leg band fixed to forward half of the rubber band to serve as tongue elevator; *j*—nails to which cannula and suction tube can be attached during operation. The rear ledge (formed from the forward upright) on which a head block for young chicks rests can be seen through the comb slot. Most of the comb slot should be forward of this ledge. Overall dimensions of the board are as shown, and a section of the board in use is shown in figure 2. (Drawn from a photograph.)

propriate pair of pegs or nails. The anterior half, at least, of the cleft in the "soft palate" should be clearly visible. The cleft is spread with the pair of fine curved forceps. The ridge of tissue connecting the anterior edges of the cleft is cut gently with the bone scraper, and immediately afterward the bottom of the cleft is scraped clean with the bone scraper; the bone appears as a glistening white area containing a small, crescent-shaped ridge enclosing a tiny, cup-shaped depression (figure 2).

Blood, mucus, or regurgitated crop contents, if present, are removed with the suction cannula, and the spreaders are inserted (figure 2). The head of the bird should now be tilted as far forward as possible without permitting the lower jaw to obscure the operating field. Drill-

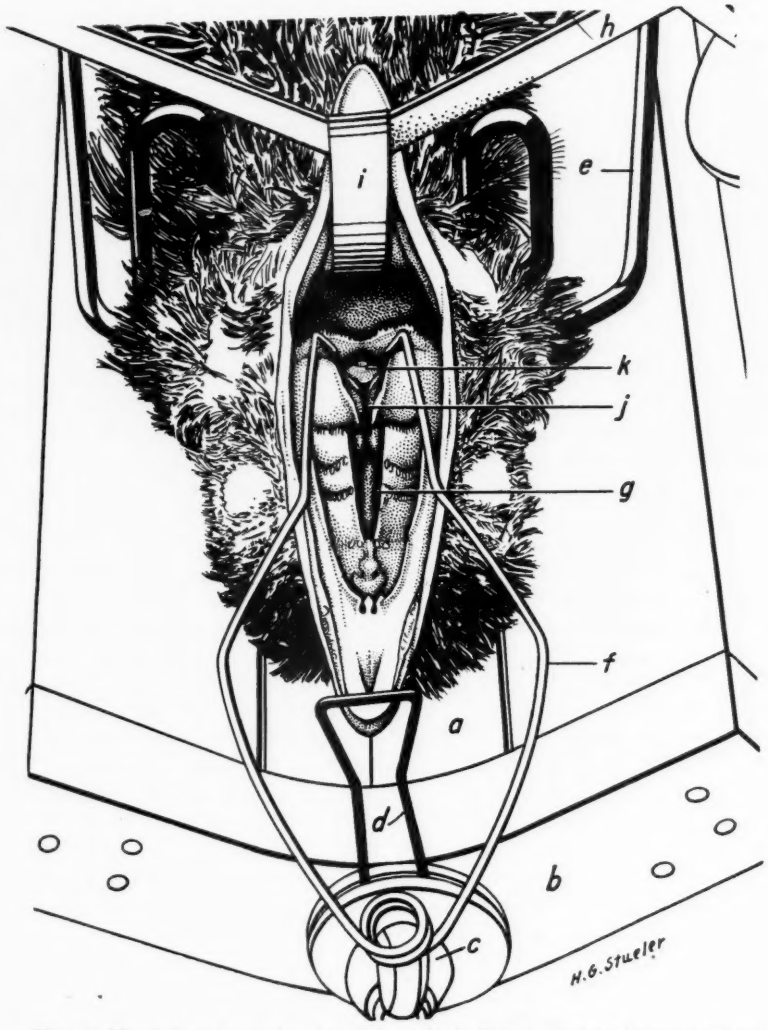

FIG. 2. Hypophysectomy by the oral method. The stage just before drilling is shown. The bird is a chick about 10–15 weeks old. *a*—head block filling comb slot in the operating board; *b*—same as in figure 1; *c*—same as in figure 1; *d*—beak holder for chicks; *e*—same as in figure 1; *f*—spring steel spreaders (the dimensions of these should be as follows—length from coil to prongs: 3 in.; length from coil to point crossed by the line from "g" in figure 2: $2\frac{3}{16}$ in.; length of prong: $\frac{1}{2}$ in.: width at widest point when prongs are in contact: $1\frac{1}{4}$ in. The prongs should be slightly concave, set at an angle of about 95° from the straight portions of the arms, and flattened at the tips. The large size of these spreaders permits their insertion into the incision without having to place one's fingers inside the mouth opening); *g*—internal nares; *h*—same as in figure 1; *j*—cleft in "soft palate," shown as it appears after being cut, and spread with the spring steel spreaders; *k*—site of drilling; the crescent shaped ridge and the cup-shape depression behind it are shown (see text). (Drawn from a photograph.)

ing is started directly on the cup-shaped depression, and continued until the hard bone has been pierced. A second hole is then started anterior to the first by a distance approximately equal to the diameter of the burr. The two drill holes should fuse, the opening be extended laterally as much as possible, and the drilling continued at an angle of about 45° from the vertical. Repeated lifting and repressing the revolving burr against the bone will help the operator to recognize the characteristic sudden "give" that signals the completion of the hole. Reference to a sagitally cut preparation of the skull of the chicken will also be of considerable help in learning the landmarks, especially the depth of the bone.

The bottom of the hole is cleaned with probe and suction cannula, and enlarged if necessary. The appearance of the pituitary and its relationship to the circle of Willis are adequately described by Mitchell (1929) and need not be gone into here. After cutting the dura with the hooked dissecting needle, gentle application of the tip of the suction cannula to the surface of the pituitary capsule will usually bring the gland away without difficulty. The bleeding that follows is stopped temporarily with cotton wool; the drill hole is then refilled with bone wax, the spreaders and rubber band removed, and the bird allowed to recover completely from the anesthetic before being replaced in its cage.

Miscellaneous Notes. Operating time. Operating time can be as little as 3 minutes, and need not be more than 10. The major factors that lengthen operating time are bleeding at the bottom or sides of the cleft in the "soft palate" misplaced drilling, and hemorrhage at the end of drilling, or on cutting the dura with the hooked needle. All of these can be avoided or minimized with practice. *Use of atropine.* Hill (1934) mentions the use of atropine to control excessive mucus secretion in the mouth. However, I have found that, without atropine, occasional aspiration of the accumulated mucus with the suction cannula keeps the operating field adequately unobstructed.

Hypophysectomy of chicks. The bird illustrated in figure 2 is a chicken about 10–15 weeks old. The operating board shown in figure 1 has been modified for use with chicks by filling the comb slot with a wooden block, using a beak holder that can be lowered to a greater extent than the one used for adults, and a rubber band retractor for the lower jaw that is about one-half as wide as that used for adults. These simple modifications make the board as easy to use for chicks as for adults.

Removal of the posterior pituitary. The term *hypophysectomy,* as used here and elsewhere in work coming from this laboratory (Rothchild, 1946, a, b, c,: 1947; Rothchild and Fraps, 1947) refers to removal of the anterior pituitary only. Removals of the posterior pituitary have been attempted only in some unpublished experiments dealing with studies on the physiology of oviposition. The posterior pituitary was burned away with a cold cautery (the Birtscher "Hyfre-

cator") after the anterior pituitary had been removed. A fine bore suction cannula was inserted into the drill hole, together with the cautery electrode, and served to keep the hole free of blood during the cauterization.

Survival. A detailed report of the survival of adult hens following hypophysectomy by this and the previously used transbuccal technique is given in the paper accompanying this one (Rothchild, 1948). In this place it will be sufficient to mention that mortality in the first week following oral hypophysectomy amounted to 12% (5 out of 42 birds), compared with an average value of 40% (52 out of 130 birds) following the transbuccal technique.

SUMMARY

A method of hypophysectomizing chickens is described, the main feature of which is the employment of the open mouth as the route by which the floor of the skull is reached. The advantages of this method over the one used previously (incision through the floor of the mouth) are its simplicity, cleanliness, and reduction in amount of time required and trauma induced.

ACKNOWLEDGMENTS

I wish to express my deepest thanks to Mr. Walter Stenhouse, the B.A.I. photographer, and to Mr. Henry Steuler of the Office of Information, U.S.D.A., for the drawings used in this paper.

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NOTES ON SURVIVAL AND BODY WEIGHT CHANGES OF ADULT HENS FOLLOWING HYPOPHYSECTOMY[1]

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INTRODUCTION

PUBLISHED information on the survival of chickens after hypophysectomy is contained in only 3 papers. Two of these deal with immature chickens (Mitchell, 1929; Nalbandov and Card, 1943), and one with adults (Hill, Corkill and Parkes, 1934). In Mitchell's study, 160 Brown Leghorn chicks ranging in age from 3 weeks to 5 months (the majority between 3–6 weeks of age) were hypophysectomized, and all in which complete removal of the gland was effected died within 2 weeks. Nalbandov and Card (1943) made a study of 60 chicks operated on at 61 days of age. Seven of these died almost immediately postoperatively, and 16 were found to have been incompletely hypophysectomized. Of the remaining 37 birds, 70% (26) died within the first 30 days, and a total of 86% within the first 60 days after operation. Hill, et al. (1934) studied survival in 30 untreated completely hypophysectomized adult chickens, and found that 24 died within the first 48 hours after operation, and the remainder by the 31st day after operation.

Some of the short-time studies from this laboratory on adult hypophysectomized chickens had indicated for some time that early mortality, though high, was not at all comparable with that obtained by Hill, et al. Since relatively long-time studies with hypophysectomized adult hens were contemplated, it was decided to obtain a more complete picture of total survival time in the absence of replacement therapy, and if possible, of the factors (such as body weight changes) that are associated with, or influence it. These studies are reported in this paper.

METHOD AND MATERIALS

Operative procedures. Two methods of hypophysectomy were used. The transbuccal technique, which has been described in detail by Hill and Parkes

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[1] A preliminary report of this study was made at the 44th annual meeting of the American Society of Zoologists (Rothchild, 1947).

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(1934), was used on 130 birds; the modification of this method using an oral approach to the pituitary (Rothchild, 1948) was used on 42 birds. The differences in survival time between the two groups are presented and discussed in the following section. In referring to the two methods from this point on, the *transbuccal* method is used for Hill's technique, and the *oral* method for my modification of it.

Birds used. White Leghorn and Rhode Island Red hens only were used. One hundred and eleven of the birds operated on by the transbuccal method were 1 to 2 years old; the 42 birds operated on by the oral method and 19 of the birds operated on by the transbuccal method (serving as controls to the oral method) were 1.5 years of age. The birds were in good health at the time of operation, but not necessarily in laying condition.

Maintenance conditions. No therapy of any kind was given after operation, the birds being returned to their cages and kept under exactly the same conditions that existed prior to operation. All birds were housed in individual cages in laying batteries, and allowed *ad libitum* access to a standard laying mash and water. The windows were open to daylight. Electric lights controlled by an automatic switch were turned on every day of the year at 6:00 A.M. and off at 8:00 P.M., so that a minimum of 14 hours of light was maintained all year round. Temperature was not controlled, except by regulating the extent of window and radiator valve openings.

Observations. Time of death was reckoned by days or weeks, the day after hypophysectomy being considered as Day 1. The birds were allowed to die spontaneously, except in a few instances in which obvious signs of approaching death were present near the close of a working day. In these cases, the birds were killed and autopsied immediately, the day of autopsy being taken as the day of anticipated death. Body weight was observed several times during the first postoperative week, then at biweekly or weekly intervals for the next 9 weeks, and at weekly or longer intervals thereafter. Observations on molt and on the weights of several internal organs were also made but are not described in this paper.

Completeness of hypophysectomy. A safety bottle was attached to the suction line, and following each operation, the line itself and the bottle were washed with tap water, and the contents of the bottle examined carefully for the removed pituitary. Notes were made on each bird's protocol sheet as to how the gland was removed, i.e., in one intact piece, in two pieces, in fragments, or not seen. In addition, at autopsy each bird's sella was examined under a pair of dissecting binoculars. In 9 cases of long-time survival (see below), one of which died in the 27th postoperative week, and the other 8 killed between the 23rd and 70th postoperative weeks, serial sections of the sellae were made,[3] and examined for pituitary rests.

In every one of the cases of spontaneous death, the pituitary was observed to have been removed in one piece at operation and/or the sella was seen to be bare of visible remnants at autopsy. Of the 9 long-time survivors whose sellae were serially sectioned, microscopic remnants of possible pituitary tissue, amounting to an insignificant fraction of the total gland, were observed in 5. Plumage, head furnishing, and body weight changes and

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[3] I am indebted to the Pathology Section of the National Cancer Institute, Bethesda Maryland, for the preparation of these sections.

extent of regression of the gonads, adrenals and thyroids of these birds differed in no appreciable respect from those seen in the long-time surviving birds that died spontaneously. I feel, therefore, that long time survival is not primarily related to pituitary rests, but is to be considered as part of the general pattern of survival following complete hypophysectomy.

RESULTS

Mortality

A. Mortality following transbuccal hypophysectomy. The numbers of deaths and mortality rates* (percentage of survivors past a given period that died in the succeeding period) per week after hypophysectomy are shown in Table 1. As noted here, 21 of the 111 birds in this

TABLE 1. NUMBER OF BIRDS DYING OUT OF THE ORIGINAL 111, AND EQUIVALENT MORTALITY RATES* PER WEEK AFTER TRANSBUCCAL HYPOPHYSECTOMY

Number weeks after hypophysectomy	Number birds dying	Mortality rate*
1	46	41.5
2	10	15.5
3	4	7.5
4	6	12.0
5	9	20.0
6	7	19.5
7	5	17.0
8	1	4.0
9**	2	8.5

* Percentage of survivors past a given period that died in the succeeding period.
** 21 birds lived after the 9th week (see text).

series lived past the 9th week. Of these one each died in the 27th, 31st and 34th postoperative weeks, two in the 35th postoperative week, and one each in the 38th, 44th, 45th, 58th, 63rd, 82nd, 85th and 88th weeks. Eight birds in perfectly good health were killed in order to obtain their sellae for serial sectioning, and for other reasons.[4] At the time of autopsy two were in the 23rd, 3 were in the 59th, and one each in the 60th, 64th and 70th postoperative weeks. (See preceding section on completeness of hypophysectomy.)

There was a possibly significant difference in the distribution of deaths within the first 9 weeks in relation to age, although both yearlings (60 birds) and 2-year olds (51 birds) showed approximately the same total mortality for this period (yearlings: 81.7%; 2-year olds: 80.4%). In the first 2 weeks after hypophysectomy 63% (38) of the yearlings, but only 35% (18) of the 2-year olds died; between the 3rd and the 9th weeks, however, 50% (11) of the surviving yearlings, and 70% (23) of the surviving 2-year olds died.

The first 3 days of the first week after hypophysectomy was in

[4] Four of these birds were killed in order to obtain their thyroids and adrenals for cytological study by Dr. F. Payne, of Indiana University.

TABLE 2. NUMBER OF DEATHS AND THE EQUIVALENT MORTALITY RATES* PER DAY OF THE FIRST WEEK AFTER TRANSBUCCAL HYPOPHYSECTOMY (111 BIRDS STARTED).

Number of days after hypophysectomy	Number birds dying	Mortality rate*
1	14	12.6
2	12	12.3
3	10	11.8
4	2	2.6
5	3	4.1
6	4	5.7
7	1	1.5

* Percentage of the survivors past a given period that dies in the succeeding period.

general the period of most severe losses (Table 2). This period becomes of special interest in comparison with the equivalent period following hypophysectomy by the oral method (see below).

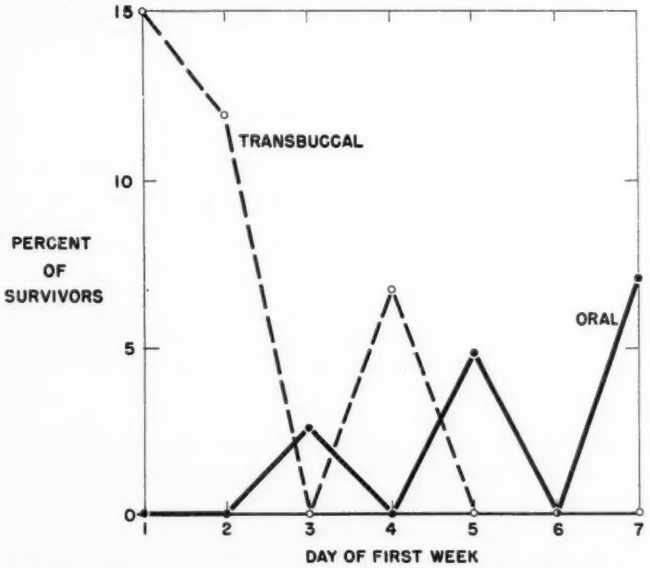

FIG. 1. Mortality rate per day, during the first week after hypophysectomy, of 42 orally hypophysectomized birds and 19 simultaneously run transbuccally hypophysectomized controls. It will be noted that the distribution of deaths following transbuccal hypophysectomy in this small series is of the same order as it is in the main series (111 birds) of transbuccal hypophysectomies (see Table 2).

B. Mortality following oral hypophysectomy. Of the 42 birds completely hypophysectomized by the oral method, the numbers dying per successive week from the first to 10th were as follows:

5, 3, 11, 7, 3, 6, 2, 1, 1, and 1.

Mortality rate for the first postoperative week was thus only 11.9%.

In contrast to this, in a series of 19 control birds, hypophysectomized by the transbuccal method at the same time as those in the oral series, the numbers lost per successive week from the first to the 9th were as follows:

6, 0, 6, 3, 0, 0, 1, 2, and 1.

The mortality rate in the first postoperative week in this group of birds was thus more than 2.5 times that of the oral series. The distribution of deaths within the first week was also quite different in the two groups (figure 1). Total mortality, however, was practically the same, and was somewhat higher than in the series of 111 transbuccal hypophysectomies described above. The differences in early mortality between all transbucally and orally hypophysectomized birds and the similarities in total mortality are shown in Table 3.

TABLE 3. ACCUMULATED PERCENTAGE MORTALITY IN THE FIRST 10 WEEKS AFTER TRANSBUCCAL AND ORAL HYPOPHYSECTOMY

Number weeks after hypophysectomy	Transbuccal hypophysectomy (130 birds started)		Oral hypophysectomy (42 birds started)	
	Number birds dead at end of week	Percent of original number dead at end of week	Number birds dead at end of week	Percent of original number dead at end of week
1	52	40	5	12
2	62	48	8	14
3	72	55	19	45
4	81	62	26	62
5	90	68	29	70
6	97	74	35	83
7	103	78	37	90
8	106	81	38	90
9	109	83	39	93
10	109	83	40	95

Body Weight Changes

The body weight changes of each of the 172 birds used in this study were plotted individually, and examined carefully for evidence of a consistent pattern or patterns. The following general statements apply to all birds, regardless of the method of hypophysectomy.

1. During the first 3 to 4 days following hypophysectomy there was a practically universal drop in body weight; in about 78% of the birds this continued throughout all the first week.

2. There was a tendency for the body weight to become stabilized at about 10–15% less than the original body weight toward the end of the first week; the stabilization of weight continued, barring death, until the end of the 9th week (figures 2 and 3).

3. Death within the first 9 weeks was preceded by a fall in body weight from the stabilized level (see paragraph 2 above), if one had been reached, or by a continued fall from the original body weight.

4. Survival past the 9th or 10th week was accompanied by a rise

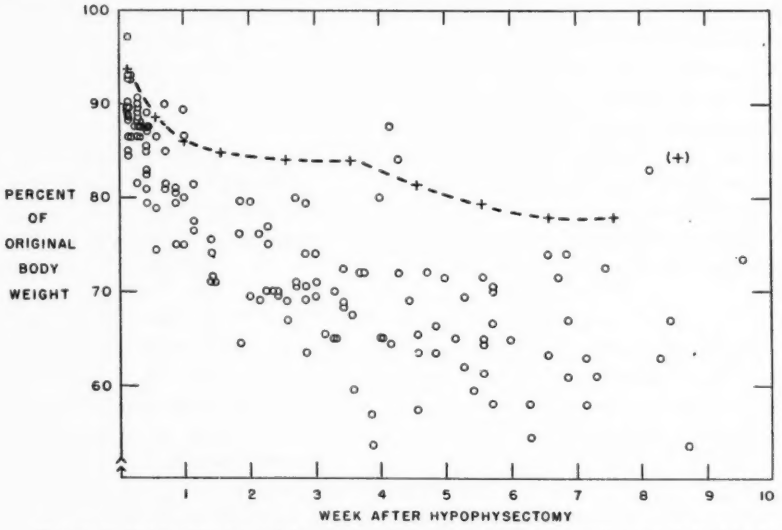

FIG. 2. Individual body weights at death (expressed as percentage of original body weight) of each bird dying within the first 10 weeks after transbuccal and oral hypophysectomy; average body weight of these short-time survivors per week after hypophysectomy is also shown (broken line and +). Body weights at death are not included in the average body weights. Average body weight shown at week 9 is placed in parentheses since only 1 bird was present in this group.

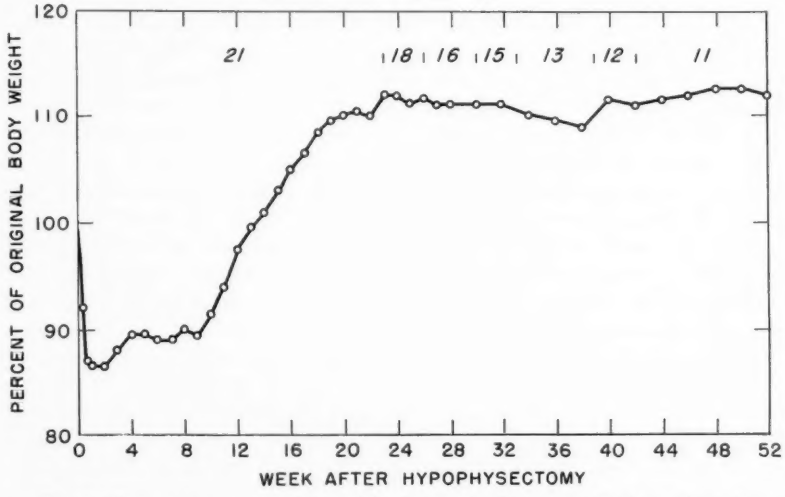

FIG. 3. Changes in average body weight during the first year after hypophysectomy of 21 transbuccally hypophysectomized birds surviving past the 9th week. Values for the average in any particular week do not include the body weights of birds showing the characteristic fall preceding death (see text). The numbers of birds included in the average are shown above the curve.

in body weight, starting roughly with the 10th week, and continuing until the 23rd week (figure 3). From this point on body weight again remained stable at about 111% of the original body weight for as long as the bird lived, but death within this period was also preceded by a fall in body weight.

5. The total amount of body weight lost up to the time of death (expressed as per cent of original body weight) increased throughout the first 30 days after hypophysectomy, and then remained fairly constant, although the amount of variation around the apparent average was quite large (figure 2).

DISCUSSION

The primary intent of the studies described in the preceding pages was to establish the general picture of survival time of adult hens, maintained without therapy of any kind, after hypophysectomy. From this standpoint, the data presented are probably as satisfactory as any. They establish the fact that a definite pattern of survival takes place. This pattern has three fairly clearly defined characteristics; a period of relatively high mortality embracing the first postoperative week and followed by a period of reduced mortality; a second period of relatively severe losses extending from about the 3rd to the 7th week, in which a major portion of all losses occur; and a period of long time survival of birds that live past the 9th–10th postoperative week. The data presented should also dispel the belief that has existed since R. T. Hill's work was done that the adult hen is unsuitable for studies involving hypophysectomy because of the excessive losses within the first 48 huors. The oral technique for hypophysectomy permits well over the majority of the birds to live without replacement therapy for about 2 weeks after operation, and there is ample time within this period for the execution of a variety of studies on the physiology of the avian pituitary.

Although the primary purpose of this study has been accomplished many interesting questions have been raised by it. The reduced mortality in the early period following oral hypophysectomy as compared with that obtained with transbuccal hypophysectomy, and the importance of this difference for determining the cause of early postoperative death; the difference in the distribution of deaths in relation to age; the cause of death in the 4th to the 7th week, and the relationship between deaths in this period and the failure to maintain a stable body weight; the fact that survival past the 9th week is practically synonymous with long-time survival, and the correlation between long-time survival and increase in body weight are some of the most interesting of these.

Some light is thrown on the question of better early survival following oral hypophysectomy by data gathered by Dr. A. Nalbandov of the University of Illinois (personal communication). He found that hypophysectomized chicks subjected to repeated short periods

of illumination, in addition to regular daylight, fed and survived much better than their controls, maintained under regular daylight alone. My orally hypophysectomized birds showed a slightly, but consistently smaller loss of body weight in the first 3 days following operation than did the transbuccally hypophysectomized birds, so it is definitely possible that better feeding contributed to the reduction of early mortality in these birds, in the same way that it contributed to the better survival of Nalbandov's chicks. The factor of reduced trauma is unquestionably present, but unfortunately, none of the data accumulated so far are conclusive enough for a clear-cut differentiation between better feeding and reduced trauma as the primary factors responsible for reduced early mortality. The same holds true for the other questions raised by this study, and it would therefore be pointless to discuss them at this time.

SUMMARY

Following hypophysectomy, adult hens tended to die most frequently in two main periods: the first week after hypophysectomy, and between the 4th–7th weeks. Roughly 80% of all birds started died by the end of the 9th week, but those remaining lived for comparatively long periods of time (27 to 85 weeks after hypophysectomy). Losses in body weight took place in practically all birds during part or all of the first postoperative week, but in the succeeding weeks up to about the 10th, a tendency for stabilization of body weight at roughly 85% of the original body weight was shown by most birds. Birds surviving past the 10th week showed a steady increase in body weight between the 10th and 25th weeks, and a stabilization of body weight at an average value of 111% of the original weight for as long as the bird lived. Death, however, at any time after hypophysectomy was preceded by a sharp drop in body weight.

Hypophysectomy was performed by two methods: using the transbuccal approach to the floor of the skull (incision through the floor of the mouth), and using the oral approach (through the open mouth). The oral approach was followed by a considerably reduced mortality in the early postoperative period, but total mortality was practically the same following either method.

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THYROGLOBULIN FORMATION IN THE THYROID FOLLICLE VISUALIZED BY THE "COATED AUTOGRAPH" TECHNIQUE

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THE UNIQUE histological structure of the thyroid has prompted a number of attempts to relate its cytological features to the process of hormone formation. However, the theories on the subject (cf. Bargmann, 1939) have left many, so far unanswered questions. For example, do the cells secrete into the circulation or in the direction of the colloid? Does this colloid merely represent the site of storage of excess thyroid hormone or does it in fact play a role in the formation of the thyroid principle? These and similar histophysiological points have remained in doubt, presumably because of the limitations of classical histology.

The use of radioactive isotopes of iodine offered a new approach to these problems. Radio-iodine may be located in sections of thyroid gland by the autographic method, which takes advantage of the action of radio-elements on a photographic emulsion. A review of the autographic results obtained to date (Gross and Leblond, 1947) showed that, within 24 hours after administration, radio-iodine was organically bound in the colloid of the thyroid follicle, both in resting and stimulated glands. However, the resolution of the earlier autographs was poor. It was, therefore, decided to reexamine the entry of radio-iodine into the thyroid of the rat by the improved "coated autograph" technique (Bélanger and Leblond, 1946; Leblond, Percival and Gross, 1948), under carefully controlled conditions of iodine intake, using intact and hypophysectomized rats. The results as reported below clarified several problems of thyroid histophysiology.

METHODS

Two similar series of experiments were carried out with essentially the same results; but only the latter will be reported in detail.

Three month old male albino rats were separated into three groups, in one of which the animals were hypophysectomized. One month later, all the animals were placed on Remington's low iodine diet (Levine, Remington and von Kolnitz, 1933) to which 5% yeast had been added. One of the two intact groups received a subcutaneous injection of 20 micrograms of iodine per rat per day. Since the diet supplied about 2 micrograms per animal per day,

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each animal in this group received a daily total of about 22 micrograms of iodine.

After 6 weeks on the diet, all the animals were given a single intraperitoneal dose of carrier-free "tracer" radio-iodine (I^{131}) as sodium iodide. In the group on the high iodine intake, the injection of the daily dose of I^{127} was discontinued 36 hours prior to the administration of radio-iodine. Each of the three groups was then divided into two subgroups, one of which was sacrificed one hour, and the other, 24 hours after the radio-iodine treatment.

The left lobe of the thyroid gland was digested in 2N sodium hydroxide by heating in a water bath and then evaporated on a watch glass for measurement of the radioactivity with the Geiger counter. The count was doubled to obtain the approximate radio-iodine uptake by the 2 lobes of the thyroid gland (Table 1).

The right lobe was left attached to the trachea, fixed in Bouin's fluid and taken through dioxane for paraffin embedding. Histological sections were cut at 5μ, mounted and dried thoroughly on a 40° electric hot plate for 2 to 3 hours. Some sections of each gland were stained with hematoxylin-eosin, and some, with Masson's light green trichrome. The sections were then coated with celloidin and photographic emulsion as indicated elsewhere (Leblond, Percival and Gross, 1948). Exposure varied from one month in the case of the hypophysectomized animals, to two hours in the case of the intact animals receiving no iodine supplement.

In order to improve the sharpness of the localization of the radio-iodine, experiments were also carried out in which the photographic emulsion was diluted 8 times with a 1% solution of Duponol C (lauryl sulphate). Under these conditions, the thickness of the emulsion coating was much reduced and the diffusion of the photographic reaction decreased (Fig. 5).

Several accessory experiments were also performed. In order to compare the thyroid stimulating action of the I-deficient diet to that of the thyrotrophic hormone, guinea pigs weighing from 150 to 200 gm. were divided into an untreated control group and an experimental group given 4 units of thyrotrophic hormone (antuitrin-T) over a period of 4 days. Then all animals received an intraperitoneal injection of tracer radio-iodine. Their thyroids were autographed and counted as above (Table 3).

At the last step of this investigation, it became important to decide whether the organic iodine elaborated by the thyroid cell was immediately released into the blood or into the colloid or simultaneously in both. For this purpose, the rate of accumulation of organic iodine in the thyroid and in the blood was compared. Eight rats weighing 150 ± 16 gm. were kept on the iodine-deficient diet for 6 weeks, and then divided into groups of two, of which one animal was left intact and the other one thyroidectomized just prior to injection. Following an intravenous injection of carrier-free radio-iodine, the groups were sacrificed 2, 7, 15 minutes and 24 hours later respectively. One thyroid lobe was autographed, the other was emulsified in saline and precipitated with an equal volume of Bouin's fluid. Both precipitate and supernatant were then counted. The blood of all animals was oxalated and centrifuged. The plasma was then divided into duplicate samples and precipitated with double volume of 10% trichloracetic acid containing 20 micrograms of iodide per cc. The precipitate was washed twice with 5% trichloracetic acid containing 10 micrograms of iodide per cc., dissolved in 0.5 N NaOH, re-

precipitated and washed as above. The supernatants and the washed precipitates were then counted under the Geiger tube (Table 4).

RESULTS

In the animals receiving a moderately high iodine intake, i.e., 20 micrograms subcutaneously daily, the thyroid follicles had a rather low epithelium. The colloid was variable in consistency and stained either red and/or green with the Masson trichrome. A fair radio-iodine

TABLE 1. THE FIXATION OF TRACER AMOUNTS OF RADIOACTIVE IODINE (I^{131}) IN THE THYROID UNDER VARIOUS EXPERIMENTAL CONDITIONS

Treatment	Approximate iodine intake (μgm./day)	% injected dose 1 hr. after injection	% injected dose 24 hrs. after injection
Low iodine diet +20 μgm. I daily	22	2.64	7.95
		2.71	16.82
		3.20	
		3.90	
		Average 3.11	Average 12.39
Low iodine diet +Hypophysectomy	2	0.23	0.41
		0.22	
		0.10	
		0.60	
		Average 0.29	Average 0.41
Low iodine diet Controls	2	33.10	72.00
		39.10	46.50
		52.20	58.10
		39.60	56.00
		Average 41.00	Average 58.15

uptake by these glands was shown with the help of the Geiger counter (Table 1). The distribution of the radio-iodine as localized on the autographs showed a definite pattern. One hour after the injection, the reactions were predominantly in the form of rings indicating the presence of iodine in the epithelium of the thyroid follicles (Figs. 1 and 3). In thin emulsion preparations viewed under high power, the individual silver granules indicative of the presence of radioactivity could be seen to occur in the cytoplasm but not in the nucleus. In the more columnar cells, the granules predominated in the apical region (Fig. 5); while in the lower cuboidal cells, the radioactivity extended throughout the cytoplasm. However, some of the follicles with a cellular reaction also had granules in the peripheral parts of the colloid. Finally, some of the more central follicles showed dot-like reactions indicating a diffusion of the radioactivity throughout the colloid (Fig. 3).

In order to estimate the size and relative numbers of the ring-like and dot-like reactions, projection drawings of unstained autographs were made at a magnification of 200 diameters. Strips through various parts of the gland were drawn at random, and a minimum of 350

follicles were counted in each section. The mean outer diameter of rings and dots was estimated by arbitrarily selecting their center, drawing the longest possible diameter and its perpendicular through each center, and averaging the length in millimeters of these two diameters. The mean inner diameter of the rings was similarly estimated by measuring two perpendicular diameters of the unreactive part (Table 2).

TABLE 2. PERCENTAGE OF FOLLICLES IN WHICH THE RADIOACTIVITY IS IN THE CELLS AT 1 HOUR AFTER INJECTION OF I^{131} (RATS GIVEN 22 MICROGRAMS OF IODINE DAILY)

	% of reactions appearing as rings	Mean outer diameter of rings	Mean outer diameter of dots	Mean inner diameter of rings	Calculated % of follicles with unreactive center*
Body of gland					
A	53	12.4	6.0	6.8	96
B	55	17.6	6.3	11.8	82
C	44	15.5	6.5	9.3	74
D	29	13.2	6.9	8.0	48
Isthmus					
A	55	9.9	5.7	5.3	102
B	48	11.7	5.9	6.8	83
C	54	16.4	8.9	8.1	109
D	63	15.3	7.2	7.6	127
Peripheral follicles					
A	71	17.2	6.5	11.0	110
B	76	18.1	6.5	12.7	108
C	67	17.1	8.0	9.5	120
D	76	15.4	7.4	9.1	128

* The calculated percentage of follicles with an unreactive center is equal to the percentage of reactions appearing as rings (1st column) multiplied by the ratio of the mean outer diameter of rings (2nd column) over the mean inner diameter of rings (4th column).

This formula is based on the assumption that the follicles are spheres. It can be shown that the ratio of the apparent to the true number of follicles with an unreactive center is equal to the ratio of the mean inner to the mean outer diameter of these follicles. The last value was not available and was replaced in the calculations by the mean outer diameter of the rings. Hence the results should be somewhat too high.

Since the production of a ring-like autograph resulted from the fact that the central portions of a follicle did not contain radioactivity, such a follicle was recognizable only when the plane of section went through the unreactive central portion. If the plane of section went through the cells of a follicle with an unreactive center, this follicle appeared as a dot. The estimated number of follicles producing a ring-like autograph will, therefore, be smaller than the true number of follicles with an unreactive center, but an approximation to the true figure may be calculated[1] (Table 2, column 5).

While the calculated results were too high, probably by 10 to 20%, the indication was that in almost all the follicles of the periphery and isthmus and in over half those of the center of the glands the radioactivity was present in the cells, while the remaining follicles of the

[1] Our thanks are due to Dr. J. Stanley, Department of Zoology, McGill University, for a mathematical study of this problem. No formal solution could be obtained.

inner regions of the gland had radioactivity extending throughout the colloid.

Twenty-four hours after injection of radio-iodine to the animals on the higher iodine intake, the autographic reactions extended to the whole colloid of practically all follicles (Figs. 2, 4 and 6). Indeed, most of the cells in these animals no longer contained radioactive material. It may be noted that the blackening of the colloid in the various follicles was quite variable, some of them showing an intense, and others, only a slight reaction (Figs. 4 and 13).

TABLE 3. THE FIXATION OF TRACER AMOUNTS OF RADIOACTIVE IODINE (I^{131}) IN THE GUINEA PIG THYROID AFTER THYROTROPHIN INJECTION

	Weight of the right thyroid lobe after fixation (mg.)	% of the injected dose found in the thyroids at 1 hr. after injection
Control	23	0.39
	31	0.83
	26	0.38
	34	0.53
	28	0.64
	15	0.37
	Average 26	Average 0.52
Thyrotrophin treated	23	3.87
	42	4.31
	46	5.19
	53	3.38
	Average 41	Average 4.19

In the hypophysectomized animals, the thyroid epithelium was flat, the lumen of the follicle, fairly large, and the colloid, rather dense and acidophilic. The radio-iodine uptake, estimated on the Geiger counter, was low (Table 1). At one hour after injection, the autographs showed the radioactive material mostly present in the cells (Fig. 7). At 24 hours, the radio-iodine was still present in the cells of most follicles, although a few follicles showed its penetration into the colloid (Fig. 8).

The thyroid glands of the control animals on a low iodine intake showed evidence of stimulation. The epithelium was increased in height; the follicular lumen was small and contained a rather thin colloid, which stained a pale green with the Masson trichrome. The radio-iodine uptake was considerable even at 1 hour after injection (Table 1). The autographs showed the radio-iodine consistently in the colloid at both one and 24 hours after injection (Figs. 9 and 12). Central collections of silver granules were found even in those follicles in which the lumen was very narrow and the colloid, so scanty as not to take up any stain. The absence of autographic reaction over the epithelium indicated that the amount of organically bound iodine present in the cells was very small or absent (Figs. 11 and 12).

Similar results were obtained with the thyroids of guinea pigs treated with thyrotrophic hormone. The radio-iodine uptake was increased (Table 3). Autographs showed the presence of the radioactivity in the colloid, not only the stimulated glands, but also in the normals.

In the experiments carried out soon after intravenous injection in iodine deficient rats, the autographs revealed the presence of radio-

TABLE 4. RADIO-IODINE (I^{131}) CONTENT OF THYROID GLAND AND TRICHLORACETIC PRECIPITATE OF BLOOD PLASMA (BLOOD PLASMA TAKEN AS 2.15% OF BODY WEIGHT)

Time after injection	Treatment	Thyroid % of inj. dose	Bouin precipitate of thyroid —% of total thyroid I^{131}	Trichloracetic precipitate of blood plasma (% inj. dose)	(% total plasma I^{131})
2 minutes	thyroidectomized	—	—	0.04	0.24
	intact	1.16	92	0.03	0.22
7 minutes	thyroidectomized	—	—	0.01	0.09
	intact	4.09	95	0.01	0.14
15 minutes	thyroidectomized	—	—	0.01	0.09
	intact	11.65	98	0.02	0.19
24 hours	thyroidectomized	—	—	—*	—*
	intact	52.50	99	0.72	78.50

* The values for the blood of the thyroidectomized animals were so low as to be within the error range of the Geiger counter background.

TABLE 5. RADIO-IODINE (I^{131}) CONTENT OF HALF-SATURATED AMMONIUM SULPHATE AND BOUIN'S FLUID PRECIPITATE OF THE THYROID GLAND

Time after injection	% of thyroidal I^{131} in Bouin precipitate	% of thyroidal I^{131} in ammon. sulph. precipitate
2 mins	96	96
7 mins	95	94
15 mins	96	93

activity in the colloid of all follicles at all time intervals. This was true even at 2 minutes after injection (Fig. 14). Quantitative measurement of the radioactivity (Table 4) showed that the thyroid glands fixed large amounts of radio-iodine. Over 90% of this iodine was precipitable with Bouin's fluid; the histological fixative used in making the autographs. However, only small quantities were observed in the trichloracetic precipitates of plasma at 2, 7 and 15 minutes after injection. These were no greater in the normal than in the thyroidectomized animals and were therefore attributed to experimental error. In contrast, 78% of the blood radio-iodine was present in the plasma precipitate of the intact animal sacrificed twenty-four hours after radio-iodine treatment. Therefore, the deposition of organic radio-iodine in the thyroid gland preceded the appearance of organic radio-iodine in the blood.

In an attempt to identity this organic compound in the gland, thyroids from animals given radio-iodine as in the previous experi-

ment were homogenized in saline. This saline extract was divided into 2 aliquots, which were then precipitated with equal volumes of saturated ammonium sulphate and Bouin's fluid respectively (Table 5). Both reagents were equally effective, precipitating over 90% of the total thyroidal iodine. Since half saturated ammonium sulphate is used to isolate thyroglobulin from the thyroid (Oswald, 1899; Rivière, Gautron and Thély, 1947), it was thus shown that the iodo-protein precipitated by Bouin's fluid was also thyroglobulin.

DISCUSSION

It should be emphasized that the amount of radioactive iodine administered to the animals consisted of a very small or "tracer" dose of iodide. This dose had an iodine content of about 0.01μg. or less, i.e., an amount of iodine too small to interfere with the normal metabolism of this element. The injected radio-iodide, mixing with the body iodide as it enters the circulation, will serve as an indicator of the behavior of iodide under physiological conditions.

Apparently the circulating iodide originates from two sources; the diet, and the breakdown of the iodinated products of the thyroid. Since a breakdown to iodide was observed after administration of di-iodotyrosine (Leblond and Sue, 1942), thyroxine (Gross and Leblond, 1947, b) and even iodo-proteins (unpublished results), this is likely to take place with the thyroid hormone after it has been secreted. The supply of iodide from the diet seems to be fairly continuous since the rat stomach contains some food at any time of the day. Both sources of iodide should provide a relatively steady supply of iodide to the circulation. Therefore, while the radio-iodine reveals a sequence of events beginning at the time of injection, the relative constancy of the iodide supply is such that the sequence of events visualized with radio-iodine repeats itself indefinitely.

The radio-iodine producing the autographic reactions must be in a chemical form that is insoluble in the reagents used for routine histological processing. Since it must be neither water nor fat soluble, it seems unlikely that it exists as iodide, or in small organic molecules such as diiodotyrosine or thyroxine. This was demonstrated at least in the case of iodide and thyroxine, which may be extracted from the tissues by several of the reagents used in histological technique (unpublished experiments). On the other hand, proteins are usually precipitated during histological fixation and thus may be retained in histological sections. Thus it was found that the thyroidal radio-iodine was precipitated equally well by both Bouin's fluid and half-saturated ammonium sulphate (Table 5). Since the latter reagent has been shown to precipitate almost all of the iodine of the thyroid as thyroglobulin (Oswald, 1899; Rivière, Gautron and Thély, 1947) it was assumed that the autographic reactions were due to the iodine present in this iodoprotein. Further, since short time intervals after injection were used, the autographs were taken to indicate the presence of *newly formed thyroglobulin*.

The sequence of events was most apparent in the moderately active glands of animals supplied with 22 micrograms of iodine daily. One hour after administration of radio-iodine, the newly formed thyroglobulin was present mostly in the epithelium of the thyroid follicle (Fig. 1). Therefore, the iodination of the proteins must have taken place in the cells, a fact in agreement with the finding in the epithelium of a peroxidase and oxidase which presumably may transform iodide into a form capable of iodinating proteins (Dempsey, 1944). Although the radioactive material seemed to be dispersed throughout the cytoplasm of the very flat cells, a clearcut localization in the apical region was visible in most other cells (Fig. 5), indicating that thyroglobulin is formed mainly in the cell apex. In our experience the same is true of colloid droplets which, when present, are located in the apex of the taller cells but are less definitely localized in lower cells. Since colloid droplets were quite rare in these sections, while the apical location of the newly formed thyroglobulin was of common occurrence the results may possibly be explained by the hypothesis that the cells contain antecedents of the colloid at or beyond the limit of microscopic visibility (Uhlenhuth, 1923; Gersh and Caspersson, 1940).

The demonstration of the sequence of events was completed by the results obtained in the 24-hour group, since at this time interval the new thyroglobulin was present in the colloid of all follicles. Obviously, the follicles which soon after injection contained the material in their cells had by now secreted it into the lumen.

In summary then, the iodide taken up from the blood by the thyroid cell reacted with the cellular proteins to yield thyroglobulin; this reaction predominating in the apical region of the cell. The iodinated protein was then rather quickly released into the colloid.

In hypophysectomized animals, the rate of formation of thyroglobulin was considerably decreased, since the radioactivity was still in the epithelium of most follicles at 24 hours after injection. It was pointed out earlier that the thyroid of the hypophysectomized rat retained the ability to collect iodide from the blood (Leblond and Sue, 1942). The thyroid cell was now found capable of building thyroglobulin and secreting it into the colloid in the absence of the hypophysis; but these phenomena took place at a much slower rate than normally.

On the other hand, the animals with stimulated thyroids, i.e., those on a low iodine intake, showed new thyroglobulin in their colloid as early as 1 hour and even 2 minutes after injection of radio-iodine (Fig. 14). Both the stimulated and resting thyroids of the guinea pig (Table 3) also showed a reaction in the colloid at 1 hour after injection. As pointed out above, these results did not necessarily mean that radioactive thyroglobulin was absent in the cells, but only that it was definitely less concentrated there than in the colloid. It is thus possible that the iodination of the proteins, and especially the deposition of the iodinated thyroglobulin into the col-

loid occurred so rapidly that at no time after injection the cells contained sufficient radioactive material to give a clearcut autographic reaction.[2] However, the results did not eliminate the less likely possibility that cells in the stimulated thyroids merely collected iodide from the blood to deposit it into the colloid where iodination would take place. At any rate, in stimulated thyroids as in the more resting glands of the iodide treated and hypophysectomized animals, the end result of the process was the accumulation of radioactive thyroglobulin in the colloid.

The evidence presented above indicated a definite polarity of the thyroid cell in its metabolism of iodine: that is to say, iodide entered the cell from the circulation, and was deposited as iodinated protein into the lumen of the follicle. This orientation of the thyroid cell toward the colloid was in agreement with the usual morphological criteria that Golgi apparatus, secretions droplets, cytocentrum and terminal bars are located between the nucleus and the secretory pole of the cell. Thus, it has been shown that terminal bars and cytocentrum were close to the membrane separating the cell from the colloid (Cowdry, 1921; Wahlberg, 1933; Bargmann, 1939). Furthermore, with the exception of flat cells, where a clearcut localization was not apparent, it was our experience as well as those of most authors to find the Golgi apparatus between the nucleus and the lumen (Okkels, 1931; Bargmann, 1939). The same might be said to colloid droplets which, in addition, increased in size as they came closer to the inner cell membrane.[3]

A careful examination of the slides obtained from the iodine-treated and control animals revealed that *all* follicles had formed some thyroglobulin as early as 1 hour after administration of radio-iodine. Similar observations were made under various experimental conditions in rats as well as in guinea pig, mouse, rabbit, man, etc. . . .(unpublished results). The only exceptions encountered were in old individuals and in hypophysectomized animals in whom some of the follicles had not fixed a detectable amount of radio-iodine after this time interval. In both cases, the extreme slowing down of the gland activity was presumably the cause of the lack of uptake. At any rate, the entry of tracer iodine in all follicles of young adult rats within 1 hour after administration indicated that *at all times all follicles were active in building up thyroglobulin and depositing it into the colloid.*

This very simple observation made it possible to eliminate the theories of thyroid function based on a "reversal" of the activity of

[2] The laws of biological precursors (Zilversmit, Entenman and Fishler, 1943) require that the bound iodine in the cells, being precursor of that in the colloid, have a higher specific activity. This condition may be fulfilled even with a low concentration of radioactivity in the cells provided their I^{127} content is low enough in relation to that of the colloid.

The presence of iodine in the epithelium has been shown by Gersh and Caspersson (1940).

[3] Biondi (1892); Uhlenhuth (1923); Severinghaus (1933); Wahlberg (1933); de Robertis (1941a, 1942); Grasso (1946); Dvoskin (1947); etc. . . .

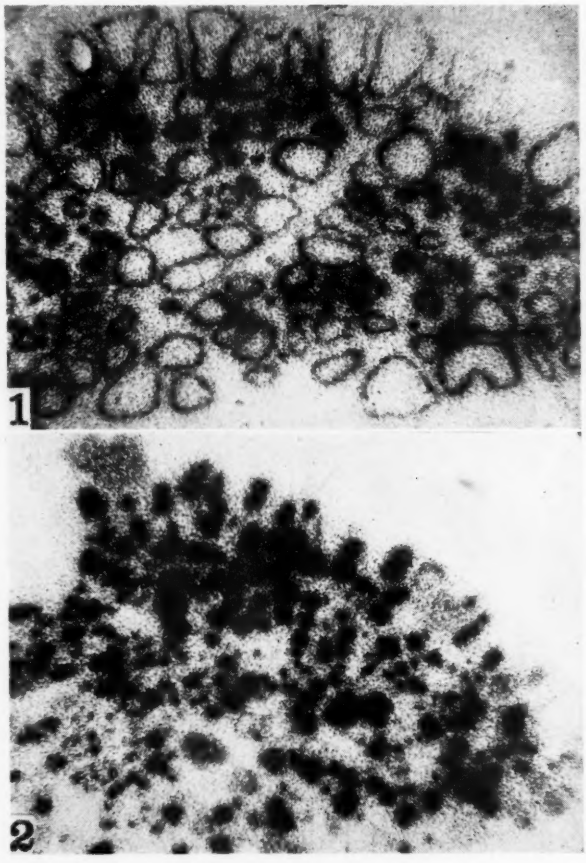

PLATE 1

FIG. 1. Unstained coated autograph of thyroid from rat treated with 20 micrograms of iodine daily. The animal was sacrificed one hour after injection of radio-iodine (Animal B, table 2). Most follicles appear as rings because of the deposition of photographic granules at the level of the epithelium. ×65

FIG. 2. Unstained coated autograph of thyroid from rat treated with 20 micrograms of iodine daily. The animal was sacrificed 24 hours after injection of radio-iodine. Practically all follicles appear as dots, because of the deposition of photographic granules over the colloid. ×65.

the follicle. According to the reversal hypothesis, the cells of a follicle are capable of secreting in the direction of the colloid at one time and, at other times, reabsorbing the colloid to secrete it into the extracellular spaces and blood vessels.[4] This theory derived support from Cow-

[4] Severinghaus (1933); Gillman (1934); Thomas (1934); Fasella (1936); Williams (1937, 1939); Feyel and Varangot (1938); de Robertis (1942). Other references may be found in Bargmann (1939).

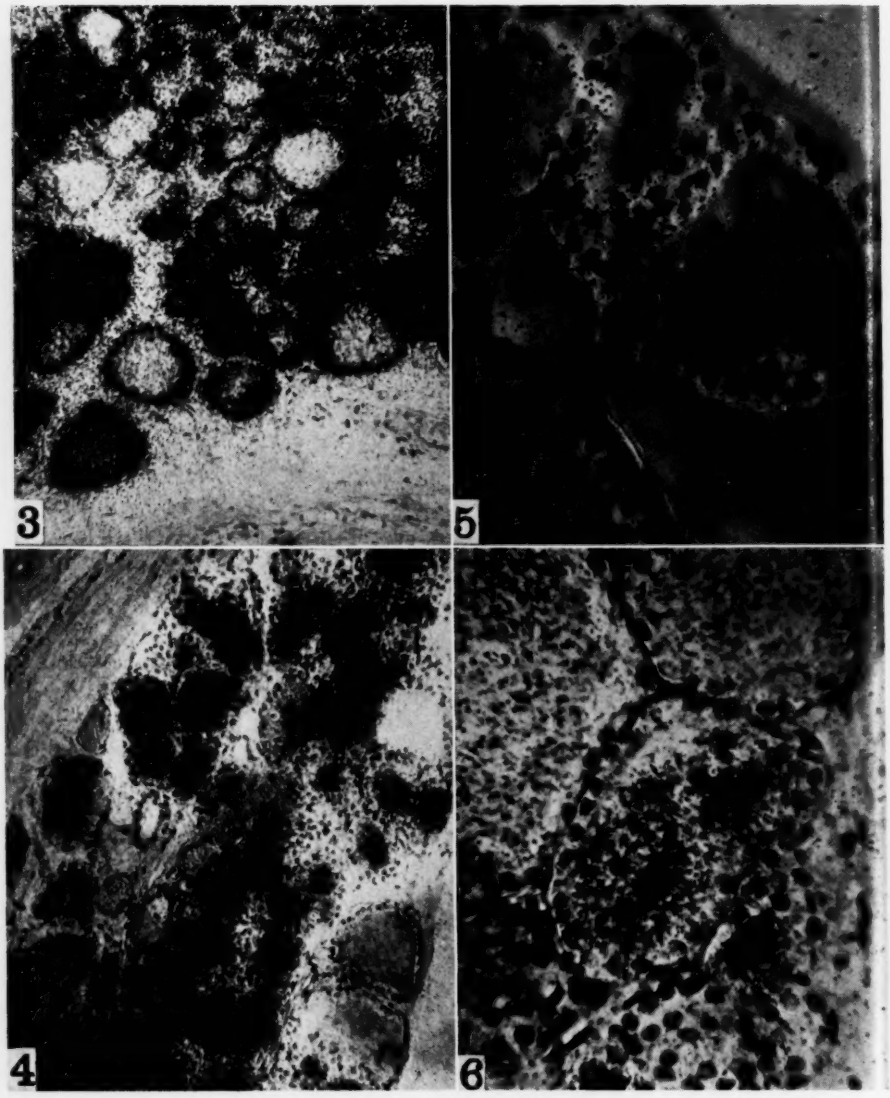

PLATE 2

H. E. stained coated autographs of thyroids from rat treated with 20 micrograms of iodine daily.

FIG. 3. The animal was sacrificed 1 hour after injection of radio-iodine (Animal C, table 2). Note both rings and dots. ×125.

FIG. 4. The animal was sacrificed 24 hours after injection of radio-iodine. The photographic reaction is present over the colloid, although its intensity varies greatly in the different follicles. ×125.

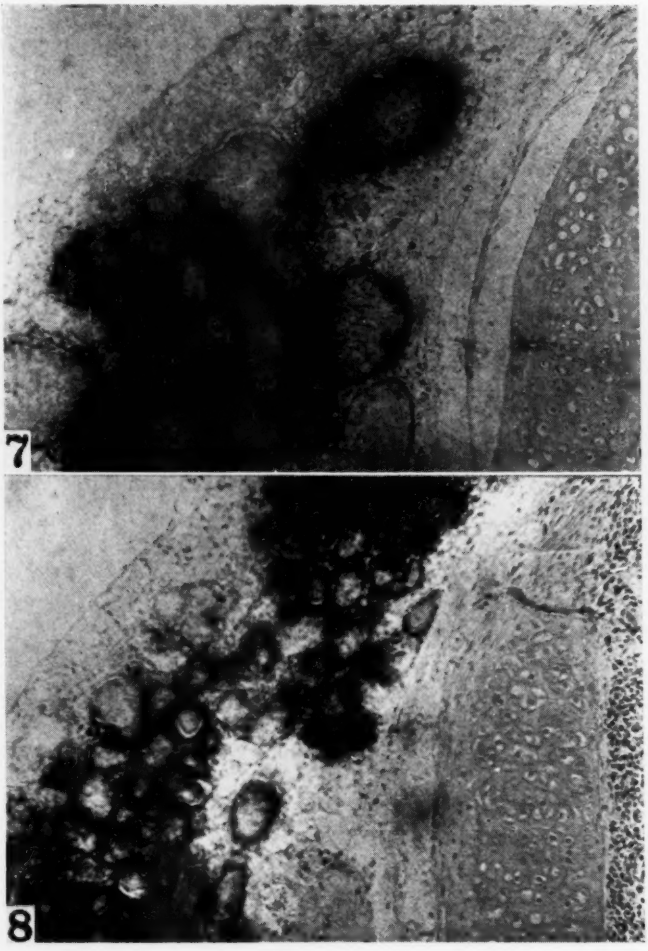

PLATE 3

H. E. stained coated autographs of thyroids from hypophysectomized rats on iodine deficient diet.

FIG. 7. The animal was sacrificed 1 hour after radio-iodine injection. The cells are thin and show a variable reaction. No reaction is apparent in the colloid. ×125.

FIG. 8. The animal was sacrificed 24 hours after radio-iodine injection. The reaction is still cellular in most follicles, although a few follicles in the upper part of the picture show the radio-iodine throughout the colloid. ×125.

FIG. 5. The animal was sacrificed 1 hour after injection of radio-iodine. A thin emulsion was used (Animal B, table 2). The amount of background fog may be estimated from the black granules in the right upper corner. A significant reaction occurs in the apical region of the cells. ×400.

FIG. 6. The animal was sacrificed 24 hours after injection of radio-iodine. Note the black granules in the colloid, but there is no more than the background fog over the cells. ×400.

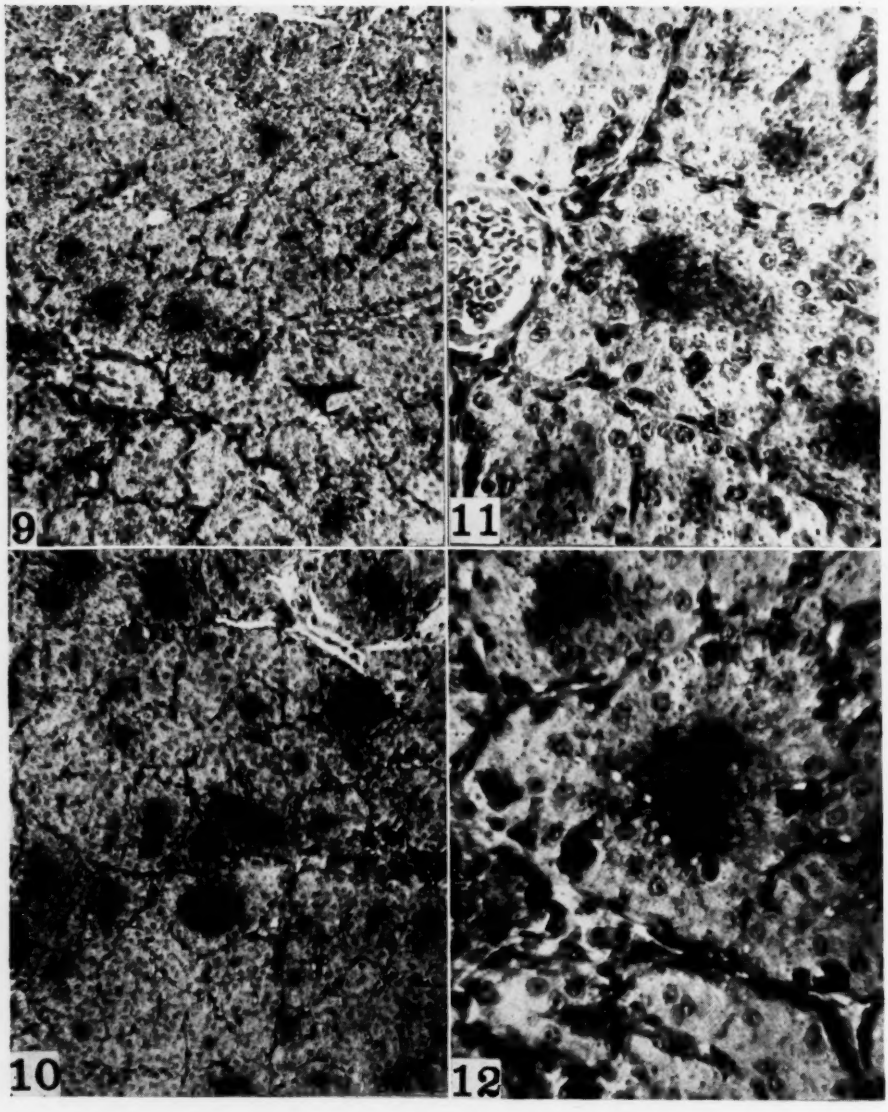

PLATE 4

H. E. stained coated autographs of thyroids of intact rats on iodine deficient diet—short exposures.

FIG. 9. The animal was sacrificed 1 hour after radio-iodine injection. The cells are large and show no reaction. Whenever the small colloid lumen happens to be in the plane of section, a reaction is apparent therein. ×125.

FIG. 10. The animal was sacrificed 24 hours after radio-iodine injection. Same as above. ×125.

FIG. 11. The animal was sacrificed 1 hour after radio-iodine injection. A larger magnification confirms that the cells do not contain enough radioactivity to produce a photographic image in excess of the background fog during the short exposure. ×400.

FIG. 12. The animal was sacrificed 24 hours after radio-iodine injection. Same as above. ×400.

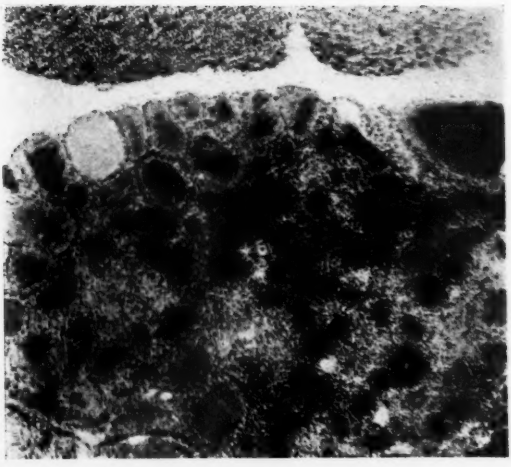

FIG. 13

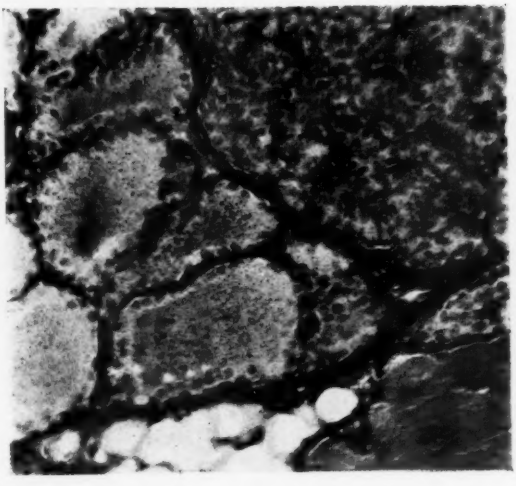

FIG. 14

FIG. 13. Masson stained coated autograph of thyroid from rat given 20 micrograms of inactive iodine daily, and sacrificed 24 hours after subcutaneous injection of radio-iodine.

Most central follicles fix a considerable amount of radioactivity in their colloid as shown by the intense black reaction. These follicles stain green, but the colour is obscured by the photographic reaction.

The red-staining follicles show only a fine stippling, indicating the presence of a lesser amount of radioactivity. ×100.

FIG. 14. Masson stained coated autograph of thyroid from rat on low iodine diet, and sacrificed 2 minutes after the intravenous injection of radio-iodine.

Photographic granules (printed as dark green dots) are visible in the colloid of the thyroid follicles. There is no reaction in parathyroid, muscle and connective tissue. ×250.

dry's finding (1922) that the Golgi apparatus, although usually apical, may be basal in some cases, and from Bensley's observation (1916) of colloid-like droplets at the base of the cells. The success of this theory was, however, not supported by more than presumptive evidence. Thus the finding of only one basal Golgi in about 500 cells in the case of Cowdry's observations (1921) was hardly indicative of colloid resorption.[5] As for Bensley's droplets (1916), in our experience they appeared watery and not colloid-like and at any rate, no valid reason existed to link them with secretion into blood velssels. Since the present observations showed that all follicles were in the process of transforming the newly entering iodine and secreting it into the colloid, none could be said to have shifted from secretion to "reabsorption" of colloid. Therefore, no support for the reversal theories was offered by these results.

Since the rings indicative of a cellular localization of neo-thyroglobulin appeared as continuous closed rings (Fig. 1), all the cells of each follicle must have been working during the short period of time—one hour—between radio-iodine injection and autopsy of the animals. Similar observations have been made previously in 2 series of rats on a "Purina Fox Chow" diet sacrificed a half hour after radio-iodine injection (see Fig. 70 in Gross and Leblond, 1946). Since these results were obtained with a tracer dose over a rather short period of time, it could be concluded that *at all times all the cells in the ring-reacting follicles* (*and by inference in all follicles*) *were continuously active*. This result made it possible to disregard the theories assuming that only some cells were active at a time, and especially the hypothesis that the so-called "colloid cells" were responsible for the secretion of the thyroid gland.[6]

Most follicles with intra-cellular radioactivity showed a fairly even distribution of the reaction throughout their epithelium and therefore, all the cells in any given follicle must have been working at a fairly similar rate. The only exception was found in the peripheral follicles which exhibited a somewhat greater deposition of material on the side closest to the center of the gland (Fig. 1). Possibly, the lesser development of the circulation under the capsule may explain the smaller reactions in this location, since a decrease in circulation should result into a decreased supply of thyrotrophic hormone, iodide and general nutrients.

It should be emphasized that the rate of iodine incorporation was different in various parts of the gland. Thus, in general, the more central follicles made and deposited thyroglobulin more rapidly than

[5] Giroud (1926) and Turchini (1927) have demonstrated that the inversion of the Golgi apparatus, such as described by Cowdry, was due to mechanical factors. Okkels (1931) confirmed the work of these authors and concluded from studies on normal and pathological glands that the Golgi apparatus changed in size with changes in cellular activity, but remained apical, a fact also noted by Wahlberg (1933).

[6] Langendorff (1889); Takagi (1922); Florentin (1928); Webster (1933); Gilmore et al. (1940); Barba (1946).

did the follicles situated just under the gland capsule or at the isthmus (Figs. 1, 3, and 13; Table 2). Besides regional differences, individual follicles showed marked variations in thyroglobulin formation (Figs. 4 and 13). In general, the follicles elaborating more thyroglobulin stained green with Masson's trichrome and may be labeled basophilic, while the less active ones usually stained red and may be labeled acidophilic (Fig. 13). This result confirmed previous observations indicating a more rapid iodine turnover in the basophilic than in the acidophilic follicles (Leblond, 1943, 1944). It may be noted however, that individual differences were less pronounced in the follicles of the stimulated glands. At any rate, each follicle had individual characteristics which at least in certain locations, such as the periphery of the gland or the isthmus, appeared definitely fixed. From these various observations, it seemed likely that each follicle maintained its own rate of formation of thyroglobulin.

So far, the excretion of the hormone out of the follicle has not been considered. One of the prevalent opinions on the subject has been that the thyroid cell secreted directly into the circulation, while the colloid lumen functioned as storage space (Bensley, 1916; Bargmann, 1939). Wegelin (1926) considered this possibility as arising only in the case of stimulated glands. However, the finding that the radio-iodine was fixed in large amounts by the stimulated thyroid gland within 15 minutes after injection (Table 4), while no radioactivity above the level of experimental error could be detected in the organic fraction of the blood, was not in favor of a direct secretion into the circulation. On the other hand, it has never been possible to detect the presence of thyroglobulin itself in the blood plasma (Lerman, 1940). It was therefore concluded that the thyroid cell was secreting only in the direction of the colloid.

Whatever the rate of entry of organic iodine into the colloid lumen, maintenance of the steady state made it imperative that it be equal to the iodine output. Furthermore, since all follicles were continuously elaborating thyroglobulin, the release of the iodinated hormone into the circulation must have taken place while thyroglobulin was being made and secreted into the colloid. An explanation of excretion fitting these requirements was afforded by the hypothesis of de Robertis (1941b; Dziemian, 1943; de Robertis and Nowinski, 1946) assuming a rapid breakdown of thyroglobulin by enzymatic proteolysis in the colloid. In agreement with this theory, a substance with thyroxine-like properties, possibly thyroxine itself (Leblond and Gross, 1948) was found in thyroid tissue resulting probably from the proteolysis of thyroglobulin. In view of their small size such fragments should diffuse out of the follice more easily than the large molecule of thyroglobulin. Indeed, thyroxine-like substances have been recovered from the plasma by Trevorrow (1939), Taurog and Chaikoff (1947) and Leblond and Gross (1948).

It may be helpful for the understanding of the follicle function to remember its entodermal origin and to venture a comparison with the

intestinal epithelium, which secretes proteins (enzymes) into the lumen in a direction consistent with the histological polarity of its cells, and at the same time resorbs from the lumen smaller molecules resulting from digestion (amino acids, . . .). Similarly, the thyroid epithelium secretes a protein (thyroglobulin) into the follicle lumen and at the same time resorbs smaller molecules probably thyroxine itself.

SUMMARY

The entry of radioactive iodine into the thyroid was observed by means of the autographic technique in the thyroids of animals kept under the following conditions: a—Rats on a low iodine intake, b—Rats receiving about 22 μgm. of iodine daily, c—Hypophysectomized rats on a low iodine intake and d—Guinea pigs receiving thyrotrophic hormone. The autographic reactions were shown to be due to radio-iodine present as thyroglobulin.

In the animals receiving 22 μgm. of non-radioactive iodine daily, the radioactivity is mostly present in the epithelium at 1 hour (Figs. 1, 3, and 5), and in the colloid at 24 hours after injection (Figs. 2, 4 and 6). In the iodine-deficient group, the deposition of radio-iodine into the colloid was much accelerated, since it was found there as early as 2 minutes after injection (Fig. 14). A rapid deposition is also found in the thyrotrophin-treated animals. In the hypophysectomized group, the deposition of radioactivity into the colloid is depressed, since the radioactive material is still present in the cells at 24 hours after injection (Figs. 7–8). These results indicate that thyroglobulin is formed in the cells and is released into the colloid, this sequence being most rapid in active thyroids and slow after hypophysectomy.

The secretion polarity of the thyroid cell in the direction of the colloid, suggested by the above data, is further demonstrated by the presence of a considerable amount of radio-iodine in the colloid at 2, 7 and 15 minutes after injection, although no significant amount of protein bound iodine can be detected in the blood at that time.

The presence of radioactivity in all follicles as early as 1 hour and in one case 2 minutes after radio-iodine injection (Fig. 14) shows that all follicles in any gland are active at all times in fixing iodine. However, the rate of iodine fixation by the various follicles is variable (Fig. 13); thus, in the rat less active follicles are located under the capsule and in the isthmus of the gland.

From the data presented, it seems likely that circulating iodide is continuously bound to protein (i.e., thyroglobulin) in the cytoplasm of the thyroid cell. This thyroglobulin is continuously deposited in the colloid of the thyroid follicle. Excretion of the thyroid hormone itself probably results from the proteolytic breakdown of thyroglobulin in the colloid and the diffusion of the resulting fragments through the epithelial wall of the follicle.

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MECHANISMS OF DESOXYCORTICOSTERONE ACTION: EFFECTS OF LIVER PASSAGE

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THE EVOCATION of hypertensive cardiovascular renal disease in experimental animals through desoxycorticosterone administration suggests that essential hypertension and its sequellae may be the result of increased activity by endogenous salt-retaining corticosteroids. Such a possibility does not necessarily imply greater production of cortical substances but might represent a defect in removal.

Detoxification of gonadal steroids is accomplished in the liver (Engel, 1944; Kochakian, Haskins and Bruce, 1944; Krichesky, Benjamin and Slater, 1943). The experimental data relative to the adrenal steroids are somewhat equivocal. The inactivity of desoxycorticosterone when orally administered (Kuizenga, Nelson and Cartland, 1940), its chemical similarity to progesterone and the identity of at least one of the detoxification products of both substances (Hoffman, Kazmin and Browne, 1943) suggest a common metabolic pathway through the liver. This supposition is supported by the decreased growth and survival rates of adrenalectomized animals when desoxycorticosterone pellets are implanted in the mesentery or spleen rather than in the subcutaneous tissues (Eversole and Gaunt, 1943). Whole cortical extract, in contrast, is active when given by mouth and relieves the symptoms of Addison's Disease, although the dose must be increased three- or four-fold (Thorn, Emerson, and Eisenberg, 1938). Furthermore adrenal cortical transplants show evidences of biologic activity when grown in the mesentery of the host animal (Eversole, Eddman and Gaunt, 1940).

The sum of these results would seem to indicate that the passage of corticosteroids through the liver results in detoxification but not to the extent of preventing the maintenance of life. Survival and growth, however, may be accomplished by the escape of small quantities of active steroids into the circulation, while large amounts would not enhance these effects proportionately. Consequently, it was considered desirable to re-evaluate the role of the liver in the removal of desoxycorticosterone by measuring the effects of hepatic passage on those actions seen at pathologic dosage levels, the intensities of which are more or less proportional to the quantity of circulating drug.

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Continuous administration of high desoxycorticosterone dosages through the use of subcutaneous implants is followed by a progressive elevation of fluid intake, complete in approximately ten days (Green and Glover, 1948; Green, Coleman and McCabe, in press). The new level of intake is related to that of controls of the same weight by a ratio fixed by the drug dosage and the degree of supplementary sodium chloride administration (Green, Coleman and McCabe, in press).

Hypertension develops in such implanted animals subsequent to the attainment of the new fluid intake level (Green and Glover, 1948; Green, 1948). When single 20 mg. pellets are used, the blood pressure climbs slowly and reaches a maximum in approximately three months. The hypertension is attended by cardiac and renal hypertrophy (Green, Coleman and McCabe, in press). Some animals die during the period of most intense blood pressure elevation. All of these manifestations are intensified if isotonic sodium chloride solution is substituted for drinking water (Green, Coleman, and McCabe, in press).

The degree to which these toxic actions are influenced by the passage of desoxycorticosterone through the liver is the subject of this report.

PROCEDURE

Single 20 mg. DCA pellets were implanted in the spleen of 5 half-grown rats of the Sprague-Dawley strain. Five control animals underwent a sham operation. Single 20 mg. pellets also were implanted in 10 animals subcutaneously. Eight normal animals served as controls for the latter group. All animals were kept in separate cages and were fed Purina Laboratory chow ad libitum. Isotonic (0.86%) saline solution was substituted for drinking water. Fluid intake was measured daily and weight weekly. At the conclusion of the 12-week period, previously shown to be the time required for the development of maximum blood pressure elevation under the circumstances described, multiple blood pressure readings were made on all surviving animals. The animals were then sacrificed and organ weights were determined.

RESULTS

The fluid intake of animals subcutaneously implanted with pellets showed a prompt rise, maximal between the first and second weeks.

TABLE 1. THE EFFECTS OF INTRASPLENIC IMPLANTATION OF DCA PELLETS ON THE BLOOD PRESSURE OF RATS AS COMPARED WITH SUBCUTANEOUS IMPLANTATION.

Drinking Fluid (0.86% NaCl)	No. of Animals	Average Blood Pressure
One 20 mg. DCA Implant Intrasplenically	4	128
One 20 mg. DCA Implant Subcutaneously	5	180
Controls:	12	114

This new level of intake, approximately twice that of the controls, was maintained throughout the experiment (Fig. 1).

By comparison, the change in the fluid consumption of animals implanted intrasplenically was minor. The maximum level of intake

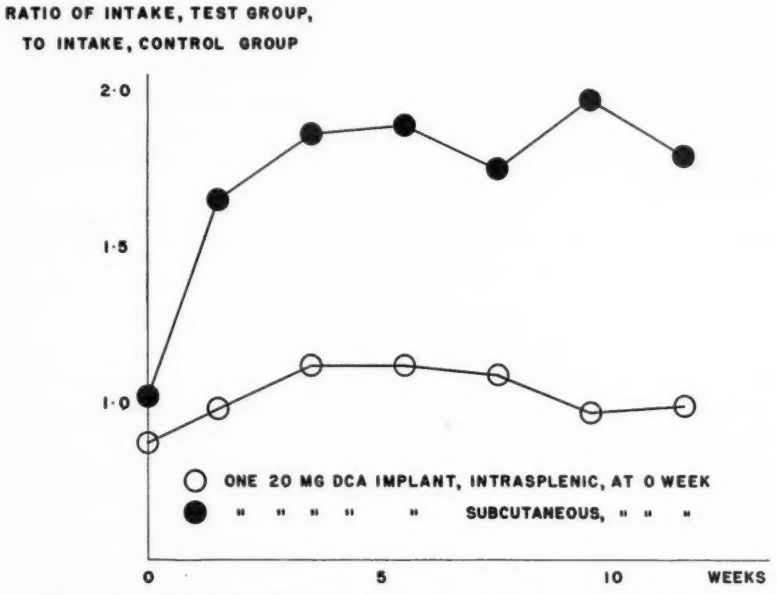

FIG. 1. The effect of intrasplenic implantation of DCA pellets on the fluid intake of rats, as compared with subcutaneous implantation. (Drinking Fluid: 0.86% NaCl).

differed from that of the sham operated group by approximately 10 per cent and was not sustained.

The average maximum blood pressure of the animals subcutaneously implanted was 180 mm. mercury as compared with 128 mm. for those implanted intrasplenically (Table 1). The average of all control blood pressures was 114 mm. mercury.

Four of the 5 animals implanted intrasplenically survived the

TABLE 2. PERCENTAGE HEART AND KIDNEY WEIGHTS IN RATS FOLLOWING INTRASPLENIC IMPLANTATION OF DCA PELLETS AS COMPARED WITH SUBCUTANEOUS IMPLANTATION.

Drinking Fluid (0.86% NaCl)	Percentage Heart Weight	Percentage Kidney Weight
One 20 mg. DCA Implant Intrasplenically	0.36	1.01
One 20 mg. DCA Implant Subcutaneously	0.92	1.29
Controls:	0.33	0.90

entire period of study. One of this group died subsequent to the operative procedure, as did one of the sham operated animals. In contrast, only one half of the subcutaneously implanted group survived the 12-week period. The others died at intervals during the second and the third months following implantation.

The ratios of heart and kidney weights to body weight of the animals implanted intrasplenically did not differ materially from those of controls (Table 2). When pellets were placed under the skin, however, these organs became uniformly hypertrophied. The percentage weights of animals in this group which died spontaneously seemed to be exaggerated in some instances by the weight loss which preceded their death.

Inspection of the implantation sites at autopsy indicated that the intrasplenic pellets had been absorbed somewhat more completely than those placed under the skin.

SUMMARY AND CONCLUSIONS

The marked alterations in fluid exchange, blood pressure, organ weight and survival which followed the subcutaneous implantation of 20 mg. DCA pellets in growing rats were reduced to minor values when pellets of the same size were implanted intrasplenically. It was concluded that the rat liver possessed a capacity for detoxifying desoxycorticosterone in quantities potentially capable of producing hypertensive disease and its organic sequellae. No evidence was secured to indicate effective use of this capacity when the drug was introduced outside of the portal circulation. The failure of the liver to protect the animal from subcutaneous DCA pellets probably was attributable to the relatively small fraction of the systemic blood flow which passed through the hepatic and portal vessels during any one circulation.

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ANTERIOR PITUITARY GROWTH PREPARATIONS: THE RELATIONSHIP BETWEEN NITROGEN STORAGE AND OTHER CRITERIA OF ACTIVITY[1]

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RESULTS of early experiments with anterior pituitary growth preparations led to a series of well correlated findings. In spite of a large increase in the metabolic rate, nitrogen storage was produced with the food intake constant. The increased caloric requirement was met by oxidation of fat (Gaebler, 1933). Storage of nitrogen was accompanied by increase in weight, and by diminished excretion of inorganic sulfate and phosphate (Gaebler and Price, 1937), suggesting synthesis of proteins, of other compounds containing nitrogen and sulfur, or of phosphorus compounds. Moreover, results in the absence of the thyroid, parathyroids, pancreas, and adrenals (Gaebler and Robinson, 1942) indicated that part of the effect on nitrogen storage might well be produced in the tissues themselves. On the basis of this experience it appeared to us that growth preparations, as distinct from purified growth hormone, might continue to be of value in studying the mechanism by which nitrogen storage is produced.

In such experiments, nitrogen storage would seem to be the logical test for activity of the preparations used, unless all assays yielded concordant results, whether based on weight, nitrogen storage, or urinary phosphate in dogs, the gain of weight in plateaued rats (Marx, Simpson, and Evans, 1942), or the epiphyseal disc response in hypophysectomized rats (Evans, Simpson, Marx, and Kibrick, 1943). In this event the determination of urinary phosphate in dogs receiving growth preparations would be the simplest assay. Gordan, Bennett, Li, and Evans (1948) obtained good correlation between the effects of purified growth hormone on weight and nitrogen storage in rats. In recent work on dogs, with assayed growth preparations supplied by several reliable firms,[2] we were at first unable to obtain correlation

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[1] The data in this paper are taken from Part 2 of a thesis submitted by one of us (P. D. B.) to the Graduate Council of Wayne University in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

[2] The authors are greatly indebted to Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis and Company, and to the late Dr. F. C. Koch, as well as to Drs. John R. Mote and Paul L. Munson of Armour and Company, for supplying assayed preparations.

between any of the five assays mentioned above. In the end, however, the value and limitations of nitrogen storage, gain in weight, and urinary phosphate as criteria of activity became fairly clear.

DIETS AND METHODS

Diet 1 consisted of beef heart trimmed free of fat, cracker meal, and bone meal. Constancy in a given experiment, and adaptability to caloric requirements of different animals, was provided for as follows: 7000 gm. of trimmed beef heart was ground, mixed with 420 gm. of bone meal, and put up in 193 gm. packs which were frozen until required. A mixture of 1630 gm. of ground trimmed beef heart and 3080 gm. of cracker meal, dried to 85 per cent of its original weight, was refrigerated in air tight cans. Each animal received daily one pack of beef heart and an amount of the mixture suited to its caloric needs. Diet 6 consisted of casein, 36 per cent; cracker meal, 36 per cent; corn oil, 20 per cent; powdered brewer's yeast, 4 per cent; and Phillips-Hart (1935) salt mixture, 4 per cent. All animals received 3 drops of haliver oil daily.

Analytical methods were the same as in earlier studies already cited. Complete nitrogen balances were done in some experiments, but nitrogen storage was usually calculated from the urine nitrogen alone, since earlier work had justified this procedure. The average of 5 or more days preceding injection was taken as the control level of nitrogen output. Since loss of nitrogen was frequently observed during the first 24 hour period following injections, irrespective of the preparation tested, this day was excluded from calculations. The period for which storage was computed ended when the urine nitrogen returned to or exceeded the control level, and was thus of variable length. Food intake was constant throughout control and experimental periods of each test.

RESULTS

The first 6 experiments shown in Table 1, hitherto unpublished, were carried out many years ago with an early type of preparation and diet 1. The amount of nitrogen stored varied greatly in individual experiments, although the diet, growth preparation, and dosage were the same throughout. However, the average amount of nitrogen stored per kilo is nearly identical for dogs 1 and 4,—0.502 and 0.498 gm. This calculation was suggested by the statement of Kochakian (1946) that the maximal rate of nitrogen storage produced by steroid hormones is directly proportional to weight in dogs. In the last 2 experiments in Table 1, the latest type of preparation and diet 6 were employed. Storage of nitrogen is again in the vicinity of 0.5 gm. per kilo. We therefore consider a preparation highly active if, when a 200 mg. dose is injected subcutaneously into dogs receiving a complete diet containing 10 gm. or more of protein nitrogen per day, it produces nitrogen storage of 0.5 gm. per kg. This may well be a maximal response. Before attempting to convert the procedure into an assay by using successively smaller amounts of the preparation, it seemed judicious to examine the relationship between nitrogen storage and other effects.

In pair fed rats receiving unpurified growth preparations (Lee and Shaffer, 1934) the excess weight which was added contained 4.8 per cent of nitrogen, as determined by actual analysis. Correlation of observed and calculated weight increase in rats receiving purified growth hormone (Gordan, Bennett, Li, and Evans, 1948) was obtained by assuming 16 per cent of nitrogen in protein and 20 per cent of protein in tissue, thus 3.2 per cent of nitrogen in the added weight. In our experiments on dogs receiving large single injections of growth preparations the amount of nitrogen stored per 100 gm. of gain in weight is much less (Table 1, second last column). In other words, gain in weight was 38 to 71 per cent greater than would be predicted on the basis of 3.2 per cent of nitrogen. Thus nitrogen stored per 100 gm.

TABLE 1. STORAGE OF NITROGEN AND INCREASE IN WEIGHT FOLLOWING SINGLE LARGE INJECTIONS OF ANTERIOR PITUITARY GROWTH PREPARATIONS

Dog No.	Weight kg.	Diet	Preparation	Date	Nitrogen Stored			Weight gain gm.
					Total gm.	Grams per kg.	Grams per 100 gm. gain in wt.	
1	21.2	1	095052A	5–12–32	14.38			453
1	20.8	1	095052A	6– 4–32	9.37			566
1	23.5	1	095052A	11–17–32	9.08			398
Average	21.8				10.94	.502	2.32	472
4	22.3	1	095052A	6–21–32	7.40			453
4	24.4	1	095052A	10–19–32	14.63			680
4	23.9	1	095052A	1–26–33	13.06			740
Average	23.5				11.70	.498	1.88	624
44	16.2	6	099783		7.54	.465	2.04	370
42	16.45	6	099816		9.94	.604	1.74	570
Average						.535	1.89	470

All injections were given subcutaneously. In each of the first 6 experiments, dogs 1 and 4 received 50 cc. of the same preparation of Antuitrin G. In each of the last 2 experiments, dogs 44 and 42 received 200 mg. of preparations also made by Parke, Davis and Co., but according to the recent procedure of Fishman, Wilhelmi, and Russell (1947). These preparations assayed 1485 and 1361 R.U. respectively.

of gain in weight has been found to vary from 3.2 to 4.8 gm. in the same species, and from less than 2 gm. to 4.8 gm. in different species, under varied experimental conditions which can be standardized.

Difficulties which came to light in the following experiments are perhaps more serious. That anterior pituitary preparations may contain substances producing nitrogen loss is shown in Table 2. The preparations injected were made as nearly as possible in the same way as the Antuitrin G used in experiments shown in Table 1. Although diet 6 had proven satisfactory (Table 1), we assured ourselves that supplementing it with 50 mg. of riboflavin daily made no difference, for diet 1 contained a great deal of beef heart, which is very rich in this factor. In Table 3 a comparison of preparations from two firms is

presented. Preparation G-731, supplied by Parke, Davis and Company, and found by them to assay 1150 R.U. per gm., was completely ineffective in dog 41 when given in 100 and 200 mg. doses. In dog 42, some nitrogen was stored, but there was loss in weight. Referring again to Table 1, it will be seen that maximal nitrogen storage was produced by preparations only 18 to 29 per cent more potent as as-

TABLE 2. SHOWING THAT ANTERIOR PITUITARY PREPARATIONS PREPARED IN THE SAME MANNER AS HIGHLY POTENT GROWTH PREPARATIONS PRODUCED NITROGEN LOSS

Preparation	Source	Dog	Weight kg.	Diet	Nitrogen Stored	
					Total gm.	Gram per kg.
099506	hog	41	15.4	6	−5.86	−0.380
099507	hog	41	15.2	6	−5.30	−0.349
099507	hog	42	16.1	6	0.86	0.053
099529	beef	42	16.0	6	2.50	0.156
099507	hog	41	15.0	6R	−8.80	−0.587
099529	beef	41	14.5	6R	0.54	0.037
099506	hog	42	15.8	6R	−1.64	−0.104
099529	beef	42	15.9	6R	−2.45	−0.154

Diet 6R consisted of diet 6 plus 50 mg. of riboflavin daily.
In each experiment, 40 cc. of anterior pituitary extract equivalent to 20 gm. of fresh anterior lobes was injected.

TABLE 3. COMPARISON OF NITROGEN STORAGE PRODUCED BY TWO PREPARATIONS OF SIMILAR POTENCY AS JUDGED BY OTHER METHODS OF ASSAY

Dog	Weight kg.	Diet	Preparation	Dose mg.	Nitrogen Stored		Weight change kg.
					Total gm.	Grams per kg.	
41	15.7	6	G-731	100	0.61	0.039	0.0
41	15.7	6	G-731	200	0.39	0.025	−0.1
42	15.4	1	G-731	200	3.42	0.222	−0.2
42	15.0	1	R-95	195	9.71	0.648	0.0

Preparation G-731 was lyophilized extract of hog anterior lobes and assayed 1150 R.U. per gm. After the above experiments, on Aug. 15, 1947, it assayed 41 epiphyseal disc units per mg. Preparation R-95 assayed 45 epiphyseal disc units per mg., ±28%, some time before, and 30 epiphyseal disc units, ±19%, on Aug. 15, 1947, after the above experiments.

sayed in the same laboratory. Since maximal nitrogen storage was produced with preparation R-95 from Armour and Company, permission was secured to send preparation G-731 to that firm for comparison with R-95 in the same laboratory. As stated in the footnote to Table 3, preparation G-731 was, in epiphyseal disc units, at least as potent as R-95. It should also be noted that the large nitrogen storage obtained with the latter preparation was unaccompanied by change in weight.

In Table 4 are recorded the results of two injections of an unsatisfactory growth preparation. Nitrogen was lost in each experiment, yet urinary phosphate fell quite definitely in both. The same anomaly was observed in 6 other experiments with various anterior pituitary preparations. In earlier experiments (Gaebler and Price, 1937), which

included phosphorus balances, there was a general parallelism between nitrogen and phosphate output in the urine. It was noted, however, that while the loss of nitrogen so often seen on the day of injection was accompanied by increased output of inorganic sulfate there was a fall in phosphate excretion on this day. The general parallelism was therefore not accepted as evidence for synthesis of nucleo-protein. It may be mentioned here that deposition of calcium phosphate, though part of the growth process, is not necessarily synchronous with nitrogen retention.

The explanation that growth preparations may contain substances

TABLE 4. DIMINISHED URINARY PHOSPHATE EXCRETION AFTER INJECTION OF ANTERIOR PITUITARY PREPARATIONS WHICH CAUSED NITROGEN LOSS

Day of experiment	Water intake, cc. per day	Urine vol., cc. per day	Weight kg.	Total urine nitrogen, gm.	Total urine inorganic phosphate, gm. as P.
1	1160	765	15.88	8.53	
2	1260	780	15.88	9.23	
3	1235	620	15.76	9.12	0.85
4	1365	680	15.88	8.95	0.81
5*	1620	1030	15.76	10.91	0.82
6	2690	1940	15.76	10.60	0.39
7	2680	1480	15.65	8.92	0.49
8	1745	1100	15.65	8.46	0.54
9	1285	1040	15.76	9.55	0.69
10–12	1210	610	15.92	9.15	0.76
13–15	1030	455	15.92	8.15	0.63
16**	1940	910	16.33	10.20	0.59
17	1400	1365	15.99	10.42	0.48
18	1470	980	15.88	9.02	0.61
19	1015	600	15.88	8.96	0.76
20	1145	610	15.99	8.56	0.74
21	950	480	15.99	9.16	0.70

* 20 cc. of aqueous alkaline hog anterior pituitary extract No. 099506 injected subcutaneously. The injection was repeated on the 6th day.

** 20 cc. of aqueous alkaline beef anterior pituitary extract No. 099529 injected subcutaneously. The injection was repeated on the 17th day. The animal received diet 6R (see footnote, Table 2) during both experiments.

which cause nitrogen loss when large single injections are given seems to cover most of the irregularities which we observed, and is supported by the data in Table 2. Since the newer preparations with which we had difficulties were much more highly purified than older preparations, we considered the alternative possibility that both nitrogen storage and gain in weight are effects of synergistic substances which can become separated. However, production of well correlated nitrogen storage and gain in weight with purified growth hormone (Gordan, Bennett, Li, and Evans, 1948) limits one at once to combined effects of growth hormone and impurities which are not chemically or biologically demonstrable. Moreover, experiments with mixtures of preparations yielded the results shown in Table 5. The thyrotropic preparation T-7210 slightly accentuated nitrogen storage when given

with G-731, and virtually abolished it when given with R-95. The pituitary gonadotrophin preparation FWP-234 produced, in one experiment, an effect very similar to that of gonadotrophin from pregnancy urine (Gaebler, 1935). Nitrogen output fell only after several days and remained below the control level for some time. In the other experiment with FWP-234 there was nitrogen loss. The potent prolactin preparation FWP-12 caused nitrogen loss in both experiments, although, in agreement with others, we have previously reported marked nitrogen storage after injection of prolactin preparations.

TABLE 5. EFFECTS OF MIXTURES OF ANTERIOR PITUITARY PREPARATIONS ON WEIGHT AND NITROGEN STORAGE

Dog	Weight	Diet	Preparations	Doses mg.	Nitrogen Stored Total gm.	Grams per kg.	Weight change kg.
42	15.4	1	G-731	200	3.42	0.222	−0.20
42	14.5	1	G-731 + T-7210	200 + 100	5.28	0.365	−0.16
42	15.0	1	R-95	195	9.71	0.648	0.00
42	14.4	1	R-95 + T-7210	200 + 100	1.26	0.087	−0.11
42	15.7	6	FWP-234	200	8.41	0.535	−0.12
42	16.2	6	FWP-12	200	−4.79	−0.296	0.00
42	16.4	6	FPW-234 + FWP-12	100 + 100	−4.62	−0.282	0.00
44	15.7	6	FWP-234	200	−4.38	−0.279	−0.17
44	15.8	6	FWP-12	200	−1.09	−0.069	−0.12
44	16.1	6	FWP-234 + FWP-12	100 + 100	−1.41	−0.087	0.00

T-7210 was a thyrotropic preparation which produced loss of both nitrogen and weight in an experiment on dog 40. G-731 and R-95 were growth preparations already described in the footnote of Table 3. FWP-234 was an Armour sheep anterior pituitary gonadotrophic preparation which the firm's assay showed to contain 159 Collip U. per mg. Other factors reported present were: thyrotropin, 0.4 chick unit per mg.; growth hormone, 0.2 epiphyseal disc units; adrenocorticotropin, 0.2% of Armour's standard; prolactin, none. FWP-12 was an Armour prolactin preparation which assayed 72 I.U. per mg. This preparation was not tested for contaminating hormones, but preparations similar to FWP-12 contained adrenocorticotrophin equivalent to 1.5% of Armour standard. Presence of appreciable amounts of gonadotrophic, thyrotropic, or growth hormone was considered unlikely.

Combination of gonadotrophin and prolactin preparations produced nitrogen loss in both experiments. In none of these tests was there notable change in weight. Weights of these large animals are subject to spontaneous fluctuation of about 0.11 kg. under our conditions. The effects of mixtures of anterior pituitary preparations on nitrogen storage appeared to be more erratic than those of single preparations, as might be expected if the irregularities are due to substances causing nitrogen loss.

SUMMARY

Growth preparations may be considered satisfactory if, when injected subcutaneously in a large single dose of 200 mg., they produce nitrogen storage of 0.5 gm. per kg. in dogs maintained on a complete diet high in protein. The gain in weight following such injections is greater than would be expected, nitrogen stored per 100 gm. of gain in weight being about 2 per cent. There is thus evidence for water

retention in excess of requirements for new tissue. Failure of correlation between nitrogen storage and other criteria of activity appears to be due to presence, in some growth preparations, of substances causing nitrogen loss, rather than to absence of any substance acting synergistically with the growth hormone to produce nitrogen storage. Phosphate output in the urine often declines even if the preparation or mixture of preparations injected causes nitrogen loss.

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THE CORRELATION OF THE CIRCULATING POLYMORPHONUCLEAR LEUCOCYTES (NEUTROPHILES) WITH THE ADRENAL ASCORBIC ACID IN THE RAT

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CLINICAL and experimental animal data reported in the past few years have indicated that there probably is a functional relationship between the activity of the adrenal cortex and the hematologic picture of individuals. Alterations in the normal white blood cell composition were reported in endocrinopathies of the adrenal cortex and the pituitary-adrenal cortex system (Balze, Reifenstein and Albright, 1946). The induction of a change from the normal white blood cell picture following ACTH in patients with adequate adrenal-cortical function has been reported by Forsham, Thorn, Prunty and Hills (1948). The hematologic changes observed in humans following alteration of adrenal-cortical function as a result of pathology or experimentally were predictable from the investigations on lower mammals by Dougherty and White (1944, 1947). The latter investigators have shown that increased activity of the adrenal cortex, particularly secretions of the 11-oxysteroid type, results in a lymphopenia specifically and a non-specific rise in the polymorphonuclear leucocytes. Selye (1940) and Reinhardt, Aron and Li (1944) had shown that injections of ACTH in rats induced a lymphopenia and a neutrophilia. Selye (1937, 1946) had reported that non-specific agents acting via the pituitary-adrenal cortical mechanism induced a lymphopenia and a polymorpho-leucocytosis.

In both the clinical and animal studies the emphasis had been placed on the relation of the adrenal cortical activity to the circulating number of lymphocytes. However, a polymorpho-leucocytosis has consistently been shown to occur with an increase in adrenal-cortical activity when these have been reported. Only recently has it been pointed out that one type of granular leucocyte, the eosinophiles, apparently vary with and are possibly indicative of adrenal-cortical activity (Forsham, Thorn, Prunty and Hills, 1948). The latter investigators also reported a neutrophilia after ACTH injections in humans but claimed this to be a non-specific effect since it occurs to some extent after ACTH in Addisonian patients.

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It has been shown that a decrease in the adrenal ascorbic acid or cholesterol content of fresh adrenal glands may be used as an indication of adrenal-cortical activity (Sayers, Sayers, Fry, White and Long, 1944; Sayers, Sayers, Lewis and Long, 1944; Sayers, Sayers, Liang and Long, 1946; Sayers and Sayers, 1946). Statistical evidence that the depletion of the ascorbic acid content of the adrenal can be used as a measure of the potency of ACTH preparations and of the activity of the adrenal cortex has recently been reported by Sayers, Sayers and Woodbury (1948). Long (1947) has presented material which indicated that the pattern of depletion (per cent change plotted against time) of the adrenal ascorbic acid was similar to the pattern of change in the circulating number of lymphocytes after ACTH injections in the rat. However, in the same paper Long quoted Dr. Oesterling's unpublished data which showed that in the scorbutic guinea pig the decrease in circulating lymphocytes was matched by a decrease in the adrenal cholesterol but no change in the already low adrenal ascorbic acid. The polymorphonuclear leucocytes were not discussed.

In view of the changes which were reported in both the lymphocyte and neutrophile picture following adrenal-cortical stimulation or adrenal-cortical endocrinopathy it seemed that a study of the relationship of these white blood cells with the adrenal ascorbic acid might elucidate the problem. Such a study appeared feasible since adrenaline had been shown to actively influence the number of circulating lymphocytes via adrenal-cortical stimulation (Long, 1947) and it had also been shown that a lymphopenia could be induced in urethanized rats which was not mediated through the adrenal gland (Dury and Robin, 1948). It was also reported that adrenaline injections in urethanized rats induced a prolonged neutrophilia. These points suggested that both adrenaline and urethane could be used as tools to actively alter the composition of the white blood cell picture and possibly yield information concerning the physiological relationship, if any, between the adrenal ascorbic acid and the circulating lymphocytes or polymorphonuclear leucocytes.

This paper will present evidence of the statistically significant correlation of the adrenal ascorbic acid with the polymorphonuclear leucocytes and the alteration of this relationship with certain drugs.

MATERIALS AND METHODS

Adult male rats of the Sprague-Dawley strain weighing 200–250 grams were used in these studies. The rats were fed Purina Chow and tap water ad lib., and lettuce was given once weekly. Total and differential leucocyte determinations were done by standard procedures on the blood obtained from the tails of the rats. The differential leucocyte values were computed after counting at least one hundred white cells on blood smears stained with Wright's blood stain. The adrenals were removed from the rats immediately after the blood was drawn for the cell counts, cleaned of fat and weighed on a

Roller-Smith precision balance to the nearest tenth of a milligram. The adrenal ascorbic acid content was determined by the method of Roe and Kuether (1943). The data were statistically evaluated by procedures suggested by Snedecor (1946). Evipal was used for anesthetic purposes.

The leucocyte picture and the adrenal ascorbic acid were studied in five different groups of rats as follows:

Group *a*. Sixteen normal intact rats in our colony were elected at random for the determinations of their adrenal ascorbic acid and leucocyte composition. These were considered the control group for this study.

Group *b*. Leucocyte counts were made before and three hours after a course of adrenaline injections in this series of fourteen rats. The adrenaline used was a 0.02% solution made up fresh for each set of injections. The rats were injected according to a procedure recommended by Long and Fry (1945) to attain adrenal-cortical stimulation. The adrenaline was injected subcutaneously at four successive hourly intervals at a dose level of 0.02 mg. per 100 gm. of body weight. Three hours after the last adrenaline injection the rats were lightly anesthetized with Evipal, blood was drawn from the tails for the leucocyte determinations and differentials, and the adrenals removed for ascorbic acid analysis.

Group *c*. All procedures were the same as above with the exception that the period between the last adrenaline injection and the final leucocyte and the ascorbic acid determinations was a total of twenty hours.

Group *d*. The rats comprising this group were given intraperitoneal injections of a 10% urethane solution every other day for a total of eight injections at a dose level of 750 mg. per kg. of body weight. The day after the last urethane injection determinations were made of the leucocyte counts, differentials, and the adrenal ascorbic acid.

Group *e*. The procedures in the rats comprising this series were exactly the same as the above group with the exception that on the day after the eighth urethane injection these rats were also given the course of adrenaline injections. Leucocyte counts, differentials, and adrenal ascorbic acid determinations were done three hours after the last adrenaline injection.

The three hour and the twenty hour periods after the last adrenaline injection was chosen as an end point on the basis of data in the literature. It had been shown that the lymphopenia and the polymorpho-leucocytosis were at a maximum approximately three hours after ACTH injections in rats; and that by approximately twenty hours after the ACTH injection these cell types were again at approximately their normal circulating number (Dougherty and White, 1944, 1947). Forsham, Thorn, Prunty and Hills (1948) had also shown that the maximum fall in the circulating lymphocytes and eosinophiles, and the maximum rise in the circulating neutrophiles occurred approximately four hours after ACTH injections in humans. The adrenal ascorbic acid had been shown to be maximally depressed one to three hours after ACTH injections and gradually approximates the normal value twenty hours after the injection (Sayers, Sayers, Liang and Long, 1946; Long, 1947).

RESULTS

The average absolute number of lymphocytes, polymorphonuclear leucocytes (in the rat these are practically all neutrophiles), the total white blood cell count per c.mm. of blood and the average adrenal

TABLE 1. THE LEUCOCYTIC PICTURE AND THE ADRENAL ASCORBIC ACID CONTENT OF NORMAL INTACT, ADRENALINE TREATED, AND URETHANIZED RATS

Animal used	Lymphocytes absolute number per c.mm.	"P"*	Polymorpho-leucocytes absolute number per c.mm.	"P"	W B C per c.mm.	"P"	Adrenal ascorbic acid mg./100 gm. fresh gland	"P"
NORMAL:								
a. Controls (16)	16,978 ± 964**	(a, b) <0.01	5,009 ± 689	<0.01	22,138 ± 1,189	>0.3	390 ± 10.35	<0.01
b. 3 hours after*** adrenaline (14)	4,984 ± 422	(b, c) <0.01	19,454 ± 2,072	<0.01	24,446 ± 2,268	>0.02	326 ± 10.0	>0.02
c. 20 hours after adrenaline (14)	10,698 ± 1,385	(a, c) <0.01	6,644 ± 1,226	>0.2	17,450 ± 2,164	>0.05	379 ± 20.79	>0.5
URETHANIZED:		(a, d) <0.01		>0.4		<0.01		>0.5
d. Controls (8)	7,664 ± 810		6,166 ± 1,213		13,831 ± 1,694		383 ± 28.84	
e. 3 hours after adrenaline (9)	2,808 ± 418	(d, e) <0.01	15,510 ± 1,787	<0.01	18,244 ± 1,912	>0.1	403 ± 32.01	>0.5
		(b, e) <0.01		>0.1		<0.05		>0.02

Number in ( ) is the number of rats in the experiment.
* "P" is the value of the Difference between the Means occurring by chance alone.
** Mean ± S.E. of the Mean
*** Adrenaline given sub-Q at a dose level of 0.02 mg. per 100 gm. of body weight for four successive hourly intervals
10% urethane given I.P. every other day at a dose level of 750 mg. per kg. for a total of eight injections.

ascorbic acid content (mg. per 100 gm. of fresh adrenal tissue) are presented in Table 1 for each of the five groups of rats used in this study. It is apparent from an inspection of Table 1 that there is no obvious functional alliance between the adrenal ascorbic acid with either the lymphocytes or the polymorphs in any single group of rats. For instance, three hours after the adrenaline treatment (Group *b*) there was a statistically significant depletion of adrenal ascorbic acid in these rats when compared with the controls; but there also was a statistically significant depression of the lymphocytes and increase in the circulating number of neutrophiles. However, when these different

TABLE 2. THE CORRELATION COEFFICIENT AND THE SIGNIFICANCE OF THE COEFFICIENT OF THE ADRENAL ASCORBIC ACID WITH THE ABSOLUTE NUMBER OF CIRCULATING LYMPHOCYTES OR POLYMORPHO-LEUCOCYTES IN THE NORMAL INTACT, THE ADRENALINE TREATED, AND UNRETHANIZED RAT

Animal used	Adrenal ascorbic acid with the absolute number of Lymphocytes		Adrenal ascorbic acid with the absolute number of Polymorphs	
	"r"*	"P"**	"r"	"P"
NORMAL:				
a. Controls (16)	0.0109	>0.05	0.5754	<0.05
b. 3 hours after*** adrenaline (14)	0.0928	>0.05	0.1488	>0.05
c. 20 hours after adrenaline (14)	0.2690	>0.05	0.6121	<0.05
URETHANIZED:				
d. Controls (8)	0.4315	>0.05	0.8899	<0.01
e. 3 hours after adrenaline (9)	0.3543	>0.05	0.0440	>0.05

* "r" is the correlation coefficient

** "P" is the value of the correlation coefficient occurring by chance alone.

*** Adrenaline given sub-Q at a dose level of 0.02 mg. per 100 gm. of body weight for four successive hourly intervals.

10% urethane given I.P. every other day at a dose level of 750 mg. per kg. of body weight for a total of eight injections.

Number in ( ) is the number of rats in the experiment.

categories of data are followed and statistically compared with each other in Groups *a*, *b*, and *c* it is apparent that the pattern of change of the adrenal ascorbic acid content is more closely followed by the pattern of change of the polymorphs than the lymphocytes. These data suggest that with adrenaline treatment the increase and return to normal number of circulating polymorphs was related to the depletion of adrenal ascorbic acid and return to normal amount. The correlation coefficient "r" and the statistical significance "P" of the coefficient was determined in each of the groups of rats for the correlation of the adrenal ascorbic acid with the lymphocytes and with the polymorpho-leucocytes (Table 2). Linear regression curves were calculated from the individual data of each group of rats and plotted according to the regression formula $Y = a + bX$ (Snedecor, 1946). These curves indicate the trend of the correlation between the adrenal ascorbic with the polymorphs or with the lymphocytes (Figures 1, 2, and 3).

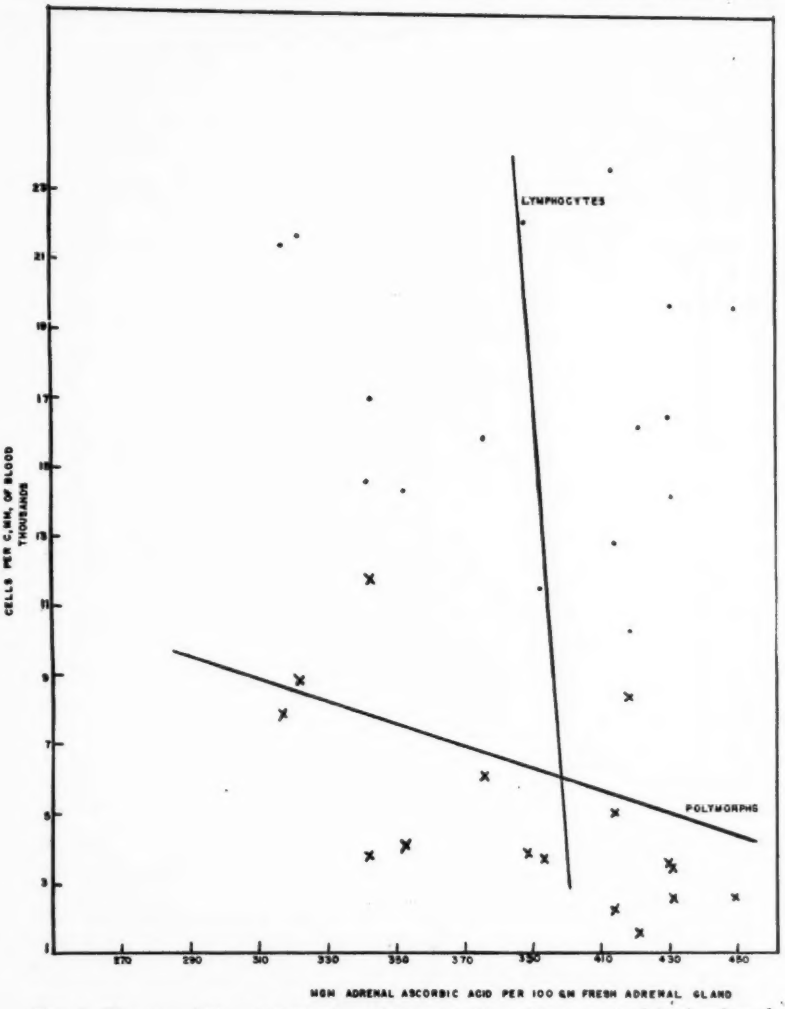

FIG. 1. The correlation distribution of the ascorbic acid content of fresh adrenal tissue with the absolute number of circulating lymphocytes and polymorpho-leucocytes per c.mm. of blood in normal intact rats. The solid lines are calculated linear regressions of the lymphocytes and the polymorphs on the adrenal ascorbic acid.

It is evident from an inspection of Figure 1 that the absolute number of circulating lymphocytes is randomly distributed and not correlated with the adrenal ascorbic acid content at the moment of autopsy. The absolute number of circulating polymorpho-leucocytes is negatively correlated with the adrenal ascorbic acid at the moment of autopsy and is statistically significant in the normal intact rat (Table 2).

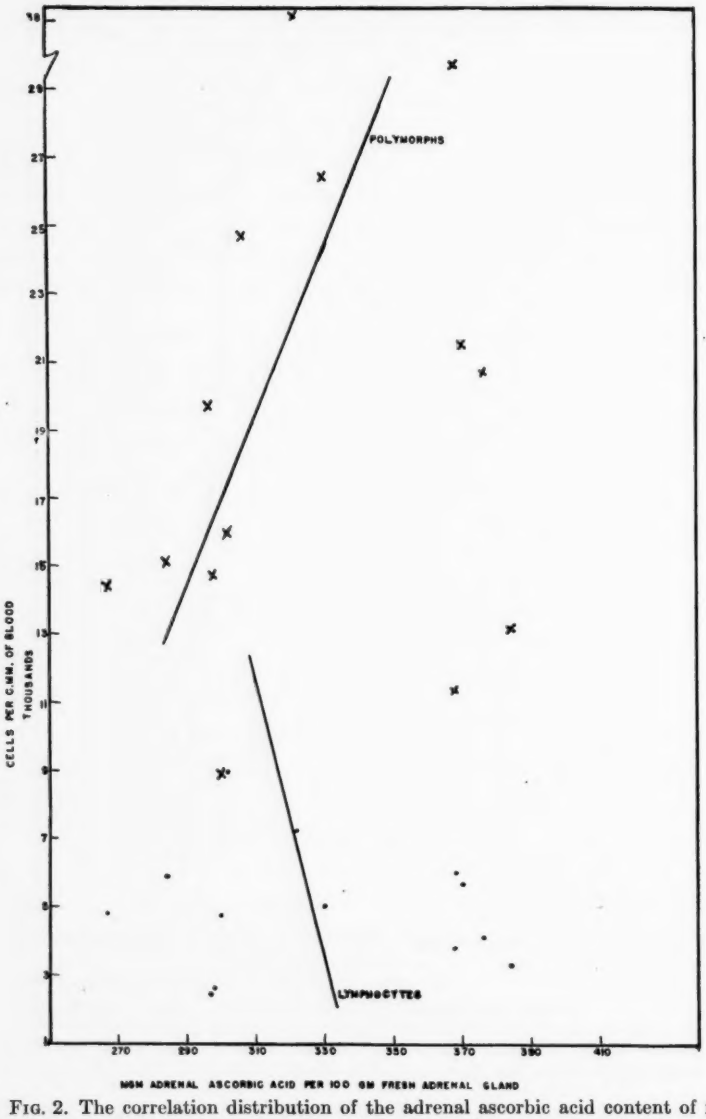

FIG. 2. The correlation distribution of the adrenal ascorbic acid content of fresh adrenal tissue with the absolute number of circulating lymphocytes and polymorpholeucocytes per c.mm. of blood in the normal rat three hours after a course of adrenaline injections. The solid lines are calculated linear regressions of the lymphocytes and the polymorphs on the adrenal ascorbic acid.

The effect of the adrenaline injections on the relationship of the adrenal ascorbic acid with the lymphocytes or with the polymorphs three hours and twenty hours after the last adrenaline injections are

shown in Figures 2 and 3, respectively. It is evident that both the polymorphs and the lymphocytes are randomly distributed with respect to the adrenal ascorbic acid three hours after the last adrenaline injection (Figure 2). The correlation coefficients are not statistically significant (Table 2). However, twenty hours after the last adrenaline injection there is a statistically significant correlation of the adrenal ascorbic acid with the polymorphonuclear leucocytes; but

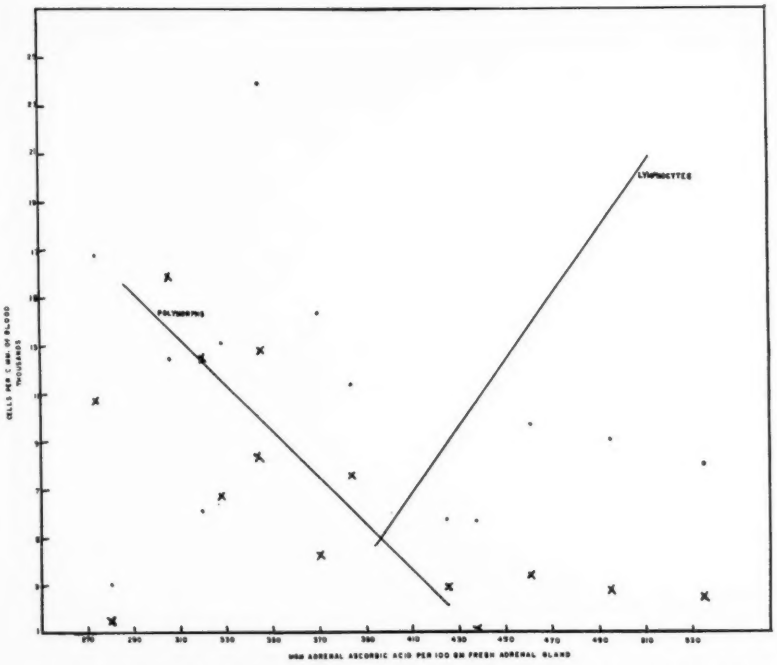

FIG. 3. The correlation distribution of the adrenal ascorbic acid content of fresh adrenal tissue with the absolute number of circulating lymphocytes and polymorpholeucocytes per c.mm. of blood in the normal rat twenty hours after a course of adrenaline injections. The solid lines are calculated linear regressions of the lymphocytes and the polymorphs on the adrenal ascorbic acid.

there is no correlation of the adrenal ascorbic with the lymphocytes (Table 2). It is also apparent from an inspection of Figure 3 that the trend of the relationship of the adrenal ascorbic acid with each of the leucocyte cell types, as represented by the linear regression curves, is toward the normal pattern found in the rat before the adrenaline injections.

A comparison of the data of the urethanized rats Group *d* with the intact controls of Group *a* yielded result-statistics which indicated that only the circulating number of lymphocytes had been altered by the urethane (Table 1). The polymorphs and the adrenal ascorbic acid

had apparently not been affected. It is noteworthy that in this group of urethanized rats that the correlation coefficient of the adrenal ascorbic acid with the polymorpho-leucocytes is statistically significant (Table 2) as was with the controls. Reference to Figure 4 shows that the linear regression curves for the adrenal ascorbic with the polymorphs and with lymphocytes are negative. It would also appear that the correlation of the adrenal ascorbic with the lymphocytes

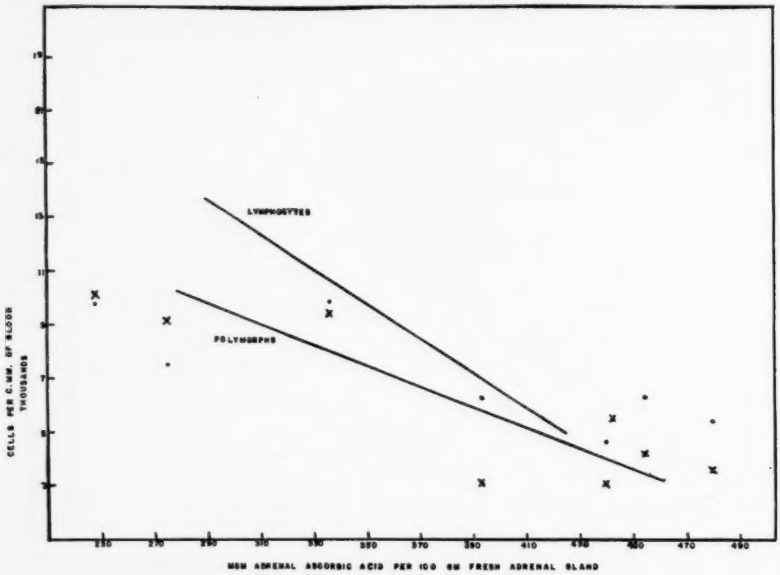

FIG. 4. The correlation distribution of the ascorbic acid content of fresh adrenal tissue with the absolute number of circulating lymphocytes and polymorpho-leucocytes per c.mm. of blood in the urethanized rat. The solid lines are calculated linear regressions of the lymphocytes and the polymorphs on the adrenal ascorbic acid.

might be significant; but statistical treatment of the data shows that this is not true (Table 2).

It had been shown by Dury and Robin (1948) that adrenaline injections in urethanized rats resulted in a neutrophilia which was still present twenty hours after the last adrenaline injection. These investigators suggested that urethane's effect was not limited to the circulating lymphocytes alone but to the polymorphs as well probably through the adrenal mechanism. In this study the rats comprising Group *e* were urethanized and then treated with adrenaline as described in the section on Methods. A comparison of the data of Group *e* with that of Groups *d* and *b* (Table 1) shows that the adrenaline had been effective in depressing the lymphocytes and increasing the circulating number of polymorpho-leucocytes. However, this apparently

did not result in a depletion of the adrenal ascorbic acid in the urethanized group of rats as had occurred in the normal rats after the adrenaline injections. The change in the correlation distribution of both the lymphocytes and the polymorphs three hours after the adrenaline injections is shown in Figure 5. The correlation coefficient of the polymorpho-leucocytes with the adrenal ascorbic acid was not statistically significant (Table 2). Since there was a statistically significant neg-

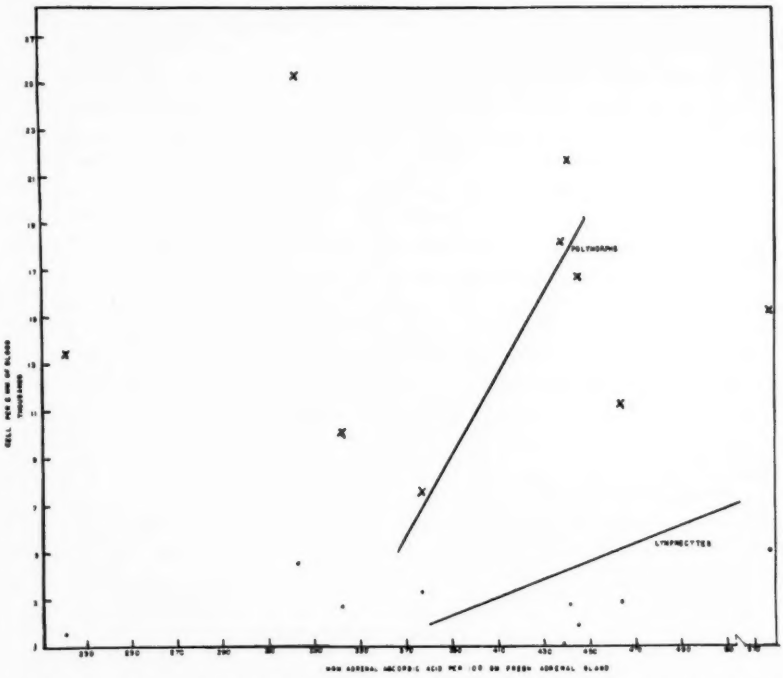

FIG. 5. The correlation distribution of the ascorbic acid content of fresh adrenal tissue with the absolute number of circulating lymphocytes and polymorpho-leucocytes per c.mm. of blood in the urethanized rat three hours after a course of adrenaline injections. The solid lines are calculated linear regressions of the lymphocytes and the polymorphs on the adrenal ascorbic acid.

ative correlation of the polymorphs with the adrenal ascorbic acid in the urethanized series of rats (Figure 4, and Table 2), the evidence from the urethanized-adrenaline treated rats is suggestive that the adrenaline injections had been effective via the adrenal-cortical mechanism. The fact that the adrenal ascorbic acid content remained at apparently normal level three hours after the adrenaline injections of these urethanized rats appears to support previous evidence of the parodoxical effect of urethane on the constituent cell types of the

white blood cell picture, and is believed to be further evidence that urethane is somehow related to its effect on the neutrophiles through the adrenal cortex.

DISCUSSION

Though the adrenal ascorbic acid has been proposed as a useful tool in the measurement of adrenal-cortical activity (Sayers, Sayers and Woodbury, 1948), it is not known exactly what role it actually has in the endocrine function of the adrenal cortex (Long, 1947). The data presented here does not assign any role to the adrenal ascorbic acid. However, it is evident from the data that there exists in the rat a functional relationship between the ascorbic acid present in the adrenal gland and the number of circulating polymorphonuclear leucocytes (in the rat this is essentially neutrophiles). This does not contradict the evidence presented by other investigators that the adrenal ascorbic acid may be used as an indicator of adrenal-cortical activity associated with lymphocyte dissolution. However, it is recognized that there is apparently no chemical relationship between the adrenals' vitamin C and the adrenal-cortical hormones.

Attention has already been called to the fact that a neutrophilia has always been reported as coexistent with a lymphopenia in the first few hours after adrenal-cortical stimulation with ACTH or adrenaline injections. The interesting aspect of the data presented here is that in the normal rat a statistically significant negative correlation has been shown to exist between the adrenal ascorbic acid and the absolute number of circulating polymorphs whereas this has not been found for the lymphocytes. This correlation of the adrenal ascorbic acid with the polymorpho-leucocytes has been supported by experimentally inducing changes in both the lymphocytes and the polymorphs simultaneously or separately with adrenaline and/or urethane and following the correlation of these white blood cells with the adrenal ascorbic acid. At no point in the experimental procedures followed was a statistically significant correlation found for the adrenal ascorbic acid with the lymphocytes; but the correlation of the polymorphs with this vitamin followed the expected pattern of relationship in adrenal-cortical stimulated animals when this had been shown to exist in the normal intact rat (Table 2). If the fact is accepted that adrenaline induces increased adrenal-cortical activity which was measured by the ascorbic acid content of the gland, then it is evident that at the point of maximum activity the physiological effect will not be correlated with the glands' ascorbic acid content. As the adrenals recoup toward a state of physiological normality there would occur a reestablishment of a normally existing regulation as indicated in this case by a correlation of the polymorphs with the adrenal ascorbic acid. An inspection of Figures 1, 2, and 3 illustrates the shifting of the polymorpho-leucocytes correlation population with relation to the adrenal ascorbic acid in the normal rat to one of complete non-correlation

three hours after adrenaline injections, and then a return to approximately the normal correlation twenty hours after adrenaline stimulation of the adrenal mechanism.

The urethanized series of rats add circumstantial and inferential evidence to the demonstrated correlation of the polymorphs with the adrenal ascorbic acid in normal rats. This is certainly illustrated by the data in Table 1 showing that the adrenal ascorbic acid is not necessarily lowered when there is a lymphopenia. Adrenaline injections can result in urethanized rats in still further depressing the number of circulating lymphocytes and also induce a neutrophilia (Table 1). These changes would indicate that the adrenal-cortex has been involved. However, in this series of rats the adrenal ascorbic acid had not been altered from the normal content. This phenomenon cannot be explained, but it is being investigated since it is suggestive of an alteration in urethanized rat of the "affinity" or "mobilization" of ascorbic acid in the adrenal gland when stimulated to increased activity.

The evidence presented in this study of the ascorbic acid correlation with the polymorphonuclear leucocytes does not mean this *a priori* has been a demonstration of the regulation of this white blood cell type by a vitamin found in high concentrations in the adrenal gland. The fact must be borne in mind that the adrenal ascorbic acid has been adequately demonstrated to be an indicator of adrenal-cortical activity. It is most probable therefore that the material in this study indicates a relationship of one of the adrenal cortex secretions to the regulation of the circulating neutrophiles. However, since the role and relationship of the vitamin C content of the adrenals to its endocrine function is not known, there is a possibility that these data are indicative of a vitamin-humoral regulation of a physiological process. This aspect of the problem is under study at present in both the rat and the guinea pig.

SUMMARY

Data have been presented which show that there is a statistically significant negative correlation of the adrenal ascorbic acid content with the number of circulating neutrophiles in the normal rat. There was no evidence of a correlation of the lymphocytes with the adrenal ascorbic acid other than the fact that following adrenal-cortical stimulation the induced lymphopenia was associated with a depletion of adrenal ascorbic acid.

The leucocytic picture was actively influenced by adrenaline injections which have been shown to be mediated via the adrenal-cortical mechanism. The adrenal ascorbic acid was found to be depleted three hours after the adrenaline injections but there was no correlation of either the lymphocytes or the polymorpho-leucocytes with the adrenal ascorbic acid. However, twenty hours after the adrenaline injections the correlation of the circulating polymorphs with the ascorbic acid had been reestablished.

Urethanization induced a lymphopenia, but did not alter the circulating number of neutrophiles nor affect the adrenal ascorbic acid. The correlation of the latter were found to be statistically significant, and the linear regression curve of the polymorphs on the adrenal ascorbic was similar to that of the normal intact rat. Urethane, however, did change the apparent effect of adrenaline on the adrenal ascorbic acid. The level of concentration of the vitamin C in the adrenal three hours after adrenaline was the same as in the normal gland although the leucocytic response was similar to that of the normal intact rat.

The data suggest that the adrenal cortex is responsible to an as yet unknown extent in the regulation of the number of circulating neutrophiles in the normal rat. The place of the adrenal ascorbic acid in this regulation is discussed. It has been suggested that urethane might be a useful tool in determining the role of the adrenals' vitamin C in the white blood cell picture of the normal rat.

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NOTES AND COMMENTS

PREGNANCY TEST WITH MALE *BATRACHIA*

THE PREGNANCY test using male *Batrachia* as reacting animals was first performed by Galli-Mainini, who in March 1947 published his first series of 179 diagnostic and control tests. This reaction, when positive, is shown by the presence of spermatozoa in a drop of the batrachian's urine, within 3 hours after the subcutaneous injection of untreated woman's urine. This new test seemed to have the advantage of specificity, speed of reaction, simplicity and economy and it has aroused the interest of many investigators, with the result that today, a year since the original publication, 27 papers on the subject have been published, with a total of 3,156 diagnostic tests and 2,108 control tests performed. In addition, a great deal of experimental work has been done on the influence of several factors on the reaction and on its mechanism. Detailed data on results will be published elsewhere and it can be summarized as follows:

Correct results: On a total of 3,156 diagnostic tests, correct positives have varied from 98.16% to 100%, according to different authors.

Specificity: The specificity is shown by 100% correct negative results on 2,108 control reactions performed by injecting non-pregnant woman's urine. Injection or implantation of more than 45 substances, purified hormones or tissues, showed that only some gonadotrophins elicit the reaction, among which chorionic gonadotrophin seems to be one of the most effective.

Comparative results with the Friedman test: On 502 simultaneously performed reactions, 42 were not coincident, of which 38 correct responses were given by the toad and 4 by the rabbit.

Speed of the reaction: Practically 100% of responses are obtained within 3 hours and 80% in two hours. Some are positive in 30 minutes.

Precocity: Correct positive reactions have been obtained in 80 tests with urine from pregnant women with less than 15 days of amenorrhea, among whom were 23 with amenorrhea from 4 to 9 days and 21 with amenorrhea of 10 days.

Toxicity: Galli Mainini's data, on a total of 3,054 diagnostic or control tests performed show 0.76% mortality as result of the urine injection.

As some investigators are under the impression that this test is the peculiar reaction of only a special type of toad, *Bufo arenarum Hensel*, or frog, *Rana pipiens*, it seems advisable to communicate that several male *Batrachia* have already been used with good results. They are: *Bufo arenarum Hensel, Bufo paracnemis, Bufo crucifer, Bufo d'Orbigny, Bufo marinus, Calyptocephalus gayi, Leptodactillus ocellatus, Odontophrynus*, and only lately, *Rana pipiens.*

We think that this reaction could be a common phenomenon of a great number of other male batrachians, providing they have a continuous spermatogenesis. Seasonal unresponsiveness can be found in some *Batrachia* with

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cyclic spermatogenesis. In these animals spermatozoa are produced only during a certain period, the testicle being at rest between the active seasons. For this reason, care should be taken in selecting an animal with continuous spermatogenesis. If so, it seems possible that many other male *Batrachia*, when injected with pregnancy urine, would respond in the same manner as the previously mentioned, thus providing a reliable test animal throughout the year. We are under the impression that toads are sturdier animals than frogs because larger quantities of urine can be injected, the mortality is less and the reaction can be repeated several times in them.

As a scarcity of bibliographic references on the subject has characterized some of the latest papers, it seems advisable to provide the available bibliography up to the present for the benefit of investigators interested in this new reaction.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21–26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be $100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

NOTES ON THE THIRTIETH ANNUAL MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirtieth Annual Meeting was held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

Forty-seven papers were presented and forty-four papers were read by title. Total registration was 458, equally divided between members and non-members. Two hundred, twenty-two were present at the dinner at which the presidential address was given by Doctor C. N. H. Long.

Actions of general interest taken by the Council are as follows:

(1) It was the decision of the Council to hold a Postgraduate Assembly in Oklahoma City, February 21–26, 1949, similar to the one which was so well received in Los Angeles in 1948.

(2) The Council approved the indexing and publication of ENDOCRINOLOGY, Volumes 1 to 40, inclusive. These will be published by Mr. Charles C Thomas, in two editions, one including the indices of Volumes 1 to 25, and the other, Volumes 26 to 40.

(3) It was the Council's decision to publish the transactions of the American Goitre Association.

(4) The Council accepted with much regret the resignation of Doctor Earl T. Engle, Chairman of the Publications Committee, and appointed Doctor Warren O. Nelson to this office. Appreciation was expressed to Doctor Engle for his work as chairman of this committee.

(5) The Committee on Registry of Endocrine Pathology was re-appointed to negotiate with the Scientific Director of the American Registry of Pathology regarding the formation of an Endocrine Registry at the Army Institute of Pathology. A sum not to exceed $750.00 was appropriated for necessary expense for one year.

(6) It was voted to appropriate $250.00 for 1949 to the National Society for Medical Research.

(7) Due to increasing cost of labor, paper and all printing materials, and the enlargement of the Journals, the dues were increased to $11.00 per year which includes subscription to either of the Journals, with a combination offer of $16.00 for both Journals.

(8) The Council voted that the thirty-first Annual Meeting be held June 3 and 4, 1949, in Atlantic City, New Jersey.

(9) A list of the 1948–49 Officers, Council and Committees follows:

Officers

President	John S. L. Browne
President-Elect	Edward A. Doisy
Vice President	James H. Means
Secretary-Treasurer	Henry H. Turner

Council

Frank A. Hartman	Roy G. Hoskins
Carl R. Moore	E. Perry McCullagh
Gregory Pincus	Fuller Albright
C. N. H. Long	Mayo Soley
Paul Starr	John S. L. Browne
Edward A. Doisy	James H. Means

Henry H. Turner

Publications Committee

W. O. Nelson, Chairman
Roy Hoskins
E. Perry McCullagh

Awards Committee

P. E. Smith, Chairman
E. C. Kendall
F. D. Lukens
Edward A. Doisy
Lawson Wilkins

Nominating Committee

John E. Howard, Chairman
E. B. Astwood
Leo T. Samuels

Committee on Registry of Endocrine Pathology

E. W. Dempsey, Chairman
Evelyn Anderson
W. U. Gardner

Membership Committee

C. N. H. Long, Chairman
George W. Thorn
Willard O. Thompson

Committee on Postgraduate Course in Endocrinology

Henry H. Turner, Chairman
John S. L. Browne
Lawson Wilkins

Committee on Nomenclature

Elmer Sevringhaus, Chairman
Hans Selye
Thomas McGavack

Consultant Committee to Food and Drug Administration

A. Kenyon, Chairman
George Sayers
J. P. Pratt

ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.